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Grouping and Occurrence of RI (Prototype RI-67) Viruses.* (22097)

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Viruses of the recently discovered RI family(1-4) which includes agents designated APC(5-8) or ARD(9) may cause undifferentiated acute respiratory disease (ARD)(10), non-*Streptococcal* exudative pharyngitis(11), primary atypical pneumonia unassociated with cold agglutinins(11), pharyngo-conjunctival fever(5-7), mesenteric lymphadenitis(12) or inapparent infections(2,4) in man. These viruses are heterogeneous anti-

genically(1,6,7,13) comprising a group of distinct serotypes(6,7) which are readily differentiable by the serum neutralization technique; however, they also elaborate a common group-specific "soluble" complement-fixing antigen which is readily separable from the virus(7,14,15) and which appears to be of little if any significance in immunity to RI disease(2,4). The seemingly ubiquitous distribution of RI agents not only in patients who are sick but also in latent or "masked" form in human adenoid or tonsil tissue(6,7, 16) poses the problem of determining which

* The majority of these observations were presented at 39th annual meeting of Am. Assn. of Immunologists, San Francisco, Apr. 11-15, 1955.

serotypes are the more important in causing respiratory illness and which are less frequently associated with human disease. The present paper summarizes the results of a study to determine the occurrence of the RI virus types in epidemics of acute respiratory illness among newly recruited soldiers in training. The antigenic grouping of the RI viruses based on tests with serum pairs from patients is also presented. Additionally, information concerning the serological response to homologous and heterologous virus serotypes in natural and experimental infections in man is summarized.

Materials and methods. Clinical. The patients with acute respiratory illness from whom blood samples and throat washings were obtained were U.S. Army personnel stationed at Fort Dix, N. J., Fort Leonard Wood, Mo. or Fort Ord, Calif.[†] during the winter of 1953, 1954 or 1955 when there was a high prevalence of infection with the RI viruses. Other articles(1-4,17) describe these epidemics. Sera were also available from volunteers who had been inoculated experimentally with the RI-67 strain of virus (18). *Laboratory. Virus isolation.* The methods employed for recovery of viruses from patients' throat washings, for propagating these agents, and for preparing complement-fixing (CF) antigens in HeLa(19) cell tissue cultures[‡] were described earlier(1). *Antigenic typing of strains.* The recovered viruses were typed by the serum neutralization procedure(1) in HeLa cell cultures employing specific rabbit antisera according to the classification system[§] of Rowe *et al.*(7). Seed virus stocks used to immunize rabbits or

employed in the neutralization tests were prepared in HeLa cell tissue cultures. Neither antiviral antisera nor antisera prepared in rabbits against uninfected HeLa culture material had a demonstrable adverse effect upon the cells in the tissue cultures when tested at a dilution of 1:4. *Serology.* Serum neutralization and CF tests with the RI viruses and patients' sera were performed according to procedures outlined(1) for use with the RI-67 strain. Serum titers were expressed as the greatest initial dilution of patient's serum which caused complete or nearly complete suppression of viral growth (neutralization) or of fixation of complement. In the neutralization tests presented in Table I, one serum pair was tested with all 11 viruses on one day. The same seed virus stocks were employed throughout.

Results. Determination of viruses important in recruit disease and antigenic grouping of strains. The acute and convalescent sera from 20 soldiers with undifferentiated acute respiratory disease (ARD) or primary atypical pneumonia of RI etiology as proved by results of CF tests were examined for increase in neutralizing antibody against 11 RI virus strains which had been isolated from patients suffering with these illnesses and also against types 1, 2, 5 and 6 of the latent viruses recovered by Rowe, Huebner *et al.* from human adenoid or tonsil tissue. The findings, presented in interpretive form in Table I, express in terms of numerical designations the relative fold-increase in serum titer of each serum pair against each virus. In the tests, each convalescent serum showed a 32-fold or greater rise in titer for at least 1 virus of the group. It is seen in the table that by far the greatest increase in antibody was against the viruses which were recovered from soldiers who were ill with respiratory disease. By contrast, the serological response to the types 1, 2, 5 and 6 latent viruses was notably weak or absent. Thus, these particular latent agents did not appear to be of etiological significance in the illnesses among the military population in this study. Further, they were of little importance in the studies of others (9) who have examined military populations.

[†] We are indebted to Lt. Col. T. O. Berge for supplying the 5 strains of virus recovered from patients at Fort Ord, and to Lt. Col. Berge, Lt. Col. H. E. Shuey, Col. L. G. Thomas, Dr. E. H. Lennette, and Capt. J. J. McCue for the serum specimens from this location.

[‡] HeLa cell cultures and tissue culture fluids employed were obtained from Microbiological Associates, Inc., Bethesda, Md.

[§] Prototype 1, 2, 3 (strain GB), 5 and 6 viruses were obtained from Dr. R. J. Huebner and type 7 virus, recently described by Berge *et al.*(20) was supplied by Lt. Col. T. O. Berge.

TABLE I. Grouping of RI Virus Strains Based on Increase in Neutralizing Antibody in Paired Sera from Patients with Respiratory Illness. Interpretive summary.

Sera from RI patient No.	Fort*	Viruses recovered from soldiers ill with respiratory disease										Increase in neutralizing antibody against RI strains					Latent viruses				
		Group A-Type 4		Group B-Type 7				Group C-Type 3		Group C-Type 3		T1+	T2	AD-71	AD-6	AD-75	T5	T6	TD-99		
		67	C-1	4-202	4-239	4-204	4-218	3-83	3-110	C-2	3-131										
4-385	Ord	5	5	2	2	2	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0
465	"	5	5	0	3	1	1	3	0	0	0	0	0	2	0	0	0	0	0	0	0
3-33	L. Wood	5	5	1	2	2	0	2	0	2	2	0	0	0	0	0	0	0	0	0	0
63	"	5	5	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
4-245	Dix	5	5	2	0	0	0	0	1	1	2	1	1	1	0	0	0	0	0	0	0
306	L. Wood	5	5	1	0	1	3	0	0	0	0	0	0	2	1	0	0	0	0	0	0
354	Ord	5	5	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
4-305	L. Wood	1	1	5	5	3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
316	"	0	2	5	3	5	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0
472	Ord	1	0	1	5	0	3	5	0	0	1	0	0	0	0	0	0	0	0	0	0
488	"	1	0	0	0	0	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0
302	L. Wood	2	1	5	3	3	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0
202	Dix	0	0	5	3	3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
239	"	0	0	4	5	3	3	3	2	0	2	0	0	0	0	0	0	0	0	0	0
4-233	Dix	5	5	3	5	3	5	3	5	4	5	3	0	0	0	0	0	0	0	0	0
3-57	L. Wood	3	3	1	5	1	2	3	2	0	2	0	0	0	0	0	0	0	0	0	0
26	"	5	5	0	0	0	0	0	3	5	3	0	0	0	0	0	0	0	0	0	0
4-237	Dix	0	0	4	4	2	4	4	4	5	4	0	0	0	0	0	0	0	0	0	0
353	Ord	0	0	5	3	3	3	4	2	2	4	0	0	0	0	0	0	0	0	0	0
3-134	L. Wood	0	0	1	5	1	0	3	1	2	0	0	0	0	0	0	0	0	0	0	0

A designation of 5 indicates the maximum increase in titer obtained with the given pair of sera. A 4 indicates a rise in titer half as great as the maximum; 3, $\frac{1}{4}$ as great; 2, $\frac{1}{8}$ as much progressing consecutively to no rise which is represented by 0.

* Fort Ord, Calif.; Fort Leonard Wood, Mo.; Fort Dix, N. J.

+ T = type.

TABLE II. Interpretive Summary of CF Test Results in Paired Sera from Patients with RI Infection. (The system for designation is explained in Table I.)

Sera from RI patient No.	Increase in complement-fixing antibody against RI strains											
	Group A-Type 4		Group B-Type 7				Group C-Type 3		T1*	T2	T5	T6
	67	C-1	4-202	4-239	4-204	4-218	3-110	GB	AD-71	AD-6	AD-75	TD-99
4-245	4	5	5	5	5	5	5	5	5	5	5	5
306	5	5	5	5	5	5	5	5	5	5	5	5
354	5	5			5	5			4	5		
4-488	4	4	4	5	5	5	4	4	4	4	5	5
202	5	5	5	5	5	5	5	5	4	4	4	4
239	3	3	4	4	5	4	5	5	2	2	0	2
4-233	5	5	5	5	5	5	5	5	5	4	5	4
3- 57	4	5	4	4	4	4	4	3	3	2	5	4
4-237	5	5	5	5	5	5	5	5	5	4	5	5
353	3	4	4	5	5	4	5	5	3	3	4	3
134	5	5	4	5	4	5	5	5	5	5	5	5

* T = type.

Table I also showed that the viruses recovered from patients with respiratory disease were antigenically heterogeneous. Nevertheless, the strains could be divided into 3 separate groups, designated A, B or C in Table I, based on the reaction with the paired sera from the patients. The first 7 pairs of sera listed showed a sharp rise in titer (designated 5) against the group A strains but little or none against the B or C agents. Conversely, the next 7 pairs of sera listed in Table I usually presented a marked increase in antibody against the B but not against the A or C viruses. The last 6 pairs of sera gave strong rises against the C viruses, and also against either the A or B strains or both. Subsequent typing of these agents with monotypic rabbit antisera demonstrated all of the A strains to belong to Rowe *et al.*'s type 4 while all of the B and C strains belonged to types 7 and 3, respectively. Thus, there was complete agreement as regards grouping of the strains either when the viruses were typed with paired sera from cases or with monotypic rabbit antisera. It is to be noted additionally

in Table I that there was greater heterogeneity within the B and C groups than among the A strains. Thus, the 2 A viruses reacted to the same extent with all of the first 7 pairs of sera while, by contrast, the B and C strains varied markedly in their reaction to the second and third groups of sera, respectively. This indicated that there was minor antigenic heterogeneity within these types which could be detected with human sera but which was not revealed in the tests with rabbit antisera.

Complement-fixation findings. The results of CF tests performed with a portion of the paired human sera shown in Table I and representative viruses are presented in Table II. The CF findings were strikingly group-specific in contrast to the highly type-specific reactions obtained in the neutralization test. Serum pair 4-239 was the notable exception in that it showed a weak reaction with the type 1, 2, 5 and 6 latent virus antigens even though the reaction with types 4, 7 and 3 was strong. This finding suggested that there may be a small degree of specificity demon-

TABLE III. Distribution of RI Virus Types among Strains Recovered from Military Patients.

Installation	Time of collection of specimens	No. of strains of type:			Total No. of strains
		3	4	7	
Fort Dix, N. J.	Jan. 11-13, '54			11	11
	" 31, '55	2	5	2	9
Leonard Wood, Mo.	" 4-9, '53	2	1	2	5
Ord, Calif.	Winter, '54	1	2	2	5*
	Total	5	8	17	30

* Viruses recovered by Lt. Col. T. Berge, Fort Baker, Calif.

TABLE IV. Distribution Neutralizing Antibody Increases for RI Viruses among 25 Patients with RI Infection.

Fold-increase in neutralizing antibody titer	No. of persons showing antibody increase against RI strains		
	Type 3 (3-110)	Type 4 (RI-67)	Type 7 (4-202)
0	15	4	7
2	2	2	3
4	1	3	3
8	2	6	4
16	2	0	
32		9	3
64	1		
128	2	1	4
256			1
No. positive (4-fold or > increase)	8	19	15

strable among the "soluble" antigens of these viruses when tested with sera from certain patients.

Distribution of virus types among the patients. Thirty RI virus strains recovered from patients with respiratory disease at Fort Dix, Fort Leonard Wood or Fort Ord were typed and the distribution of the various serotypes in these military camps is shown in Table III. It is seen that types 3, 4 and 7 only were represented and that all 3 types were present in all 3 camps in spite of their wide geographic separation. The 1954 outbreak at Fort Dix was of special interest in that it was largely if not entirely due to type 7 virus alone(17). To obtain further information about the occurrence of these agents, the paired sera from 25 patients with RI infection selected from the same populations in the same epidemics from which the viruses in Table III were recovered were tested for increase in neutralizing antibody against type 3, 4 and 7 viruses. Table IV, which summarizes the increases in antibody against the strains, showed that 8 patients developed a significant rise in titer against type 3 virus, 19 against type 4 and 15 against type 7. Although one cannot always be confident of the infecting type when the serological response alone is measured (see Table V,) the data are sufficiently clear to permit the conclusion that types 4 and 7 virus were the most common in these particular military outbreaks.

Homotypic and heterotypic response to infection. The findings in tests to determine

the type-specificity of the serological response to natural infection among patients in whom the infecting type was definitively established by recovery of virus are summarized in Table V. The increases in neutralizing antibody were predominantly homotypic although the heterologous response to type 3 or 4 virus in the last three cases in the table was as large or larger than that against the type 7 strains which were recovered from the throat washings. Patient 4-246, in whom the heterologous response was 16 times greater than the homologous, provided the most outstanding example of this experience. The serologic findings and the identity of the isolate were determined on several occasions. The possibility cannot be excluded, however, that the patient may have been infected with both type 3 and 7 virus within the time period for collection of the serum specimens. Similar tests to determine the heterotypic response in patients infected experimentally with the RI-67 strain of type 4 virus by several routes are summarized in Table VI. It is noteworthy that the neutralizing antibody response in these individuals was entirely type-specific and there was no rise to either heterologous type. It is of further interest that the artificial infection gave no heterotypic booster effect even in the patients who had antibody against the heterologous virus prior to inoculation with the RI-67 agent.

Discussion. It is evident that the RI viruses recovered from patients with respiratory illness exhibited marked antigenic heterogeneity when tested by the neutralization method with paired sera from cases and that these strains were clearly differentiated into three serological groups. These groups, which correspond to types 3, 4 and 7 as determined by tests with monotypic rabbit antisera(7), showed further minor antigenic heterogeneity within types 3 and 7, the significance of which remains to be determined.

All the viruses recovered from patients belonged to type 3, 4 or 7 and the antibody response in the persons with respiratory disease was against these 3 types primarily. There was little or no antibody produced against the type 1, 2, 5 or 6 latent viruses(7,16) recov-

TABLE V. Homotypic and Heterotypic Neutralizing Antibody Response in Patients with RI Infection from Whom Virus Was Recovered.

Case No.	Virus isolate type	Serum specimen	RI group CF	Antibody titer		
				Type 3	Type 4	Type 7
55- 11	3	A*	0†	0†	0	0
		C	80	512	0	8
55- 33	3	A	0	0	2	0
		C	5	128	2	0
55- 9	4	A	0	0	0	0
		C	160	8	32	0
55- 8	4	A	0	2	0	0
		C	20	8	32	2
4-229	7	A	0	0	0	0
		C	80	0	8	512
4-239	7	A	0	ND†	0	2
		C	20	0	0	512
4-202	7	A	0	ND	0	0
		C	160	0	0	32
4-207	7	A	0	ND	0	0
		C	160	0	8	32
55- 6	7	A	0	0	0	0
		C	20	0	8	32
4-204	7	A	5	ND	0	0
		C	80	0	2	8
4-215	7	A	0	ND	0	0
		C	40	0	2	8
4-201	7	A	0	0	2	0
		C	20	0	2	8
4-218	7	A	0	ND	0	0
		C	80	0	8	8
4-233	7	A	0	ND	0	0
		C	80	0	128	32
4-246	7	A	0	0	0	0
		C	10	128	0	8

* A = acute, and C = convalescent specimen collected 3 or 4 wk later.

† 0 titer in CF test = less than 1:5 and in neutralization test = less than 1:2, the lowest serum dilutions tested.

‡ ND = Tests not done.

ered from human adenoid or tonsil tissue in tissue culture and these latter agents did not appear to be of etiological significance in any of the cases studied. All 3 serotypes 3, 4 or 7 were present in troops in each of the 3 camps and it is to be noted that the personnel in these installations were recruited from the eastern (Fort Dix), central (Fort Leonard Wood) and western (Fort Ord) regions of the United States. Types 4 and 7 virus appeared most often among the soldiers while type 3 was less prevalent. One may speculate that the less frequent occurrence of type 3 might have been related to immunity resulting from prior infection with this agent which is known to cause epidemics in children(5,8).

Patients infected naturally with type 3, 4

or 7 virus usually exhibited antibody increase of low order against at least one other type even though antibody against the heterotype was absent from the acute phase specimen. By contrast, volunteers given type 4 virus (RI-67) by the respiratory route or parenterally gave a homotypic response only even in persons who had antibody initially against another type. The reason for this difference was not evident although it may be related to the fact that the infection in the volunteers was inapparent or associated with minor illness(18).

The present findings are of importance in the laboratory diagnosis of RI infections and in any consideration of immunologic control of the disease. For routine laboratory diag-

TABLE VI. Homotypic and Heterotypic Neutralizing Antibody Response in Volunteers* Given RI-67 Virus.

Patient No.	Age and sex	Virus inoc. route	Serum specimen†	CF‡ RI group	Antibody titer		
					Neutralizing‡		
					Type 3	Type 4	Type 7
1	71 ♂	Nebulizer	Pre	0	2	0	8
			Post	0	2	32	8
5	29 ♂	Nebulizer & intraderm.	Pre	0	8	0	0
			Post	160	8	128	0
6	53 ♂	<i>Idem</i>	Pre	0	2	8	0
			Post	5	2	128	0
7	54 ♂	"	Pre	0	0	8	0
			Post	40	0	512	0
8	43 ♀	"	Pre	0	0	0	0
			Post	160	0	512	0
9	63 ♂	Intranasal & intraderm.	Pre	0	0	2	0
			Post	10	0	32	0
10	21 ♂	Intramuse.	Pre	0	0	0	2
			Post	80	0	512	2
11	55 ♀	Intramuse. & intraderm.	Pre	0	2	0	0
			Post	40	2	512	0
12	43 ♀	Intramuse.	Pre	0	2	2	0
			Post	20	2	128	0
13	53 ♂	Intramuse. & in'tumor	Pre	0	2	0	0
			Post	0	2	128	2

* Details of the volunteer experiments will be reported elsewhere(18).

† Pre-inoculation specimen collected immediately prior to administration of virus and post-inoculation sample taken 8 to 45 days later.

‡ 0 titer = less than 1:2 in the neutralization test; less than 1:2 in the pre-inoculation CF samples and less than 1:5 in the post-inoculation specimen.

nosis of RI infections, the CF test is the method of choice since a single antigen suffices to detect antibody increase in all cases. Where additional information of the serotype is important, the specific neutralization test with types 3, 4 and 7 virus should be employed or the virus itself should be recovered and typed. As regards immunization of newly recruited soldiers, one would certainly wish to induce resistance against types 3, 4 and 7 viruses.

Summary. Viruses of the RI family which caused acute respiratory illness among soldiers at Fort Dix, N. J., Fort Leonard Wood, Mo., and Fort Ord, Calif. during the last few years were separated into 3 groups based on neutralization tests with paired sera from cases. These 3 groups of agents corresponded to serotypes 3, 4 and 7 of Rowe *et al.* determined by tests with monotypic rabbit antisera. Latent virus types 1, 2, 5 and 6 which were recovered originally from tonsil or adenoid tissues by others did not appear to be of etiologic importance in the mili-

tary illnesses. The CF test results, in contrast to the neutralization findings, were markedly group-specific. The importance of strain heterogeneity in relation to diagnosis and immunity is discussed.

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Multiplication of Virulent Poliovirus in Capuchin Monkey Kidney Cultures Without Microscopically Observed Cytopathogenicity.* (22098)

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Previous work from this laboratory demonstrated that the South American ringtail monkey (*Cebus capucina*) has a limited susceptibility to poliovirus *in vivo*(1). Recent experiments showed that the Type 2 Y-SK strain, highly cytopathic for rhesus kidney cultures, was without demonstrable effect in capuchin cultures(2). The present report extends the earlier findings in demonstrating that Type 1 human strains can multiply in epithelial cultures of capuchin kidney, even though no obvious cytopathic changes could be observed. In the course of these experiments, material became available to determine whether capuchin cultures, which supported virus growth with no apparent cell destruction, could act as a selective medium for the propagation of non-neurotropic variants of poliovirus[‡](3-8).

Materials and methods. Four Type 1, four Type 2 and three Type 3 strains were employed, both as stool suspensions of patients ill with poliomyelitis and as the first passage

material from infected rhesus kidney cultures (in which they were all markedly cytopathogenic). The procedures in use in this laboratory for growing cultures, for isolation and titration of viruses have been reviewed recently(9). In order to test for survival of virus under the conditions of the experiments, control tubes were inoculated and incubated together with the capuchin cultures. The control tubes contained medium alone (without cells) or rhesus cells that had been killed by freezing. Normal controls of uninoculated capuchin cultures were also included. For each test 0.1 ml of fluid was inoculated into a series of 8 to 10 monolayer cultures of capuchin kidney cells and 0.9 ml of lactalbumin hydrolysate medium(9) was added. The cultures were allowed to incubate for various times; at the end of each incubation period, the fluids were harvested, and fresh medium was added if the incubation was to be continued. Titrations were carried out in rhesus kidney cultures, using 8 tubes per dilution, since the capuchin cultures showed no apparent cytopathic alterations. The plaque technic was carried out according to the method of Dulbecco and Vogt(10). Tests for neurotropic properties of the Type 1 strains were carried out by inoculating the first or second passage capuchin culture fluid into

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TABLE I. Passage of Type 1 Poliovirus in Cultures of Capuchin Monkey Kidney.

Strain	Passage in capuchin cultures	Cone. of TC fluid inoc.	Incubation period (days)	TCD ₅₀ titer in rhesus cultures (per ml)
Easton -15	1	10 ⁰	14	5.0*
	2	"	14	4.1*
	3	10 ⁻¹	7	>5.0
		10 ⁻²	7	4.6
		10 ⁻³	7	2.0
		"	11	>4.0
	4	10 ⁻³ †	7	1.7
		" †	11	3.1
		" †	18	4.0
	5	10 ⁰	2	4.6
NE-23	1	10 ⁰	14	4.3*
	2	10 ⁻¹	7	5.8
		10 ⁻³	7	>4.0
		"	11	>5.0
	3	10 ⁻⁴ †	7	3.0
		" †	11	4.3
		" †	18	4.4
NE-34	4	10 ⁰	2	4.7
	1	10 ⁰	14	4.0*
		10 ⁻¹	7	5.4
		10 ⁻³	7	>4.0
		"	11	>5.0
	3	10 ⁻⁴ †	7	2.8
		" †	11	2.8
		" †	18	3.6
	4	10 ⁰	2	5.1

* Virus could not be recovered from tubes containing medium alone or rhesus cells killed by freezing, which were inoculated with the same amount of virus and incubated for the same period of time as these capuchin cultures.

† These dilutions were made of the fluids harvested from the cultures of the previous passage which had been inoculated with virus diluted a thousandfold.

rhesus monkeys, 2 intracerebrally (0.5 ml) and 2 intraspinally (0.1 ml). The 4th or 5th passage material was tested in 6 monkeys for each of the strains.

Results. The Type 1 strains grew readily in trypsinized capuchin kidney cultures, although no cytopathic changes could be observed in the microscope. The infected cultures looked exactly like the uninoculated controls. No evidence could be obtained to show that Type 2 and 3 strains multiplied under the same circumstances. The results of the titrations and the number of passages for three Type 1 strains are given in Table I. There are 3 lines of evidence to show that multiplication of virus occurred:

1. All the passages were made by the addition of 0.1 ml of virus or harvested fluids into

0.9 ml of medium in the culture tubes. Successive dilutions for the 5 passages of strain E-15 were 10¹, 10¹, 10⁴, 10⁴, and 10¹, making the final dilution of the original material 10¹¹. The titer of virus was never greater than 10⁵ TCD₅₀/ml, and since virus was present in the final fluid at a titer of 10^{4.6}, virus multiplication must have occurred. This is supported by the failure to obtain virus from the control tubes (no living cells present) incubated during the first two passages together with the capuchin cultures.

Similar considerations apply to strains NE-23 and NE-34; these also showed the presence of virus at relatively high levels in the final culture fluids, which represented a dilution of 10¹¹ of the original fluids.

2. Multiplication was shown as well by the increase of virus during a single passage. When capuchin cultures were inoculated with less than 10 TCD₅₀ of strain E-15 in its second passage, fluids with a titer of greater than 10^{4.0} were obtained, indicating that at least a thousandfold increase in virus concentration had occurred. This type of virus increase was obtained several times and also with the other Type 1 strains.

3. Virus propagation was also demonstrated by titrations of fluids taken at different times from the culture tubes. With passage 3 of the E-15 strain, fluids harvested from the cultures inoculated with 10 TCD₅₀ yielded a titer of 10^{2.0} at the end of 7 days and one greater than 10^{4.0} at the end of 11 days of incubation. This increase of virus concentration with time is shown also in passage 4: the titers of the harvested fluids were 10^{1.7}, 10^{3.1}, and 10^{4.0} at the end of 7, 11, and 18 days, respectively. Similar results were obtained with the other two strains.

To determine whether all the capuchin cells in the culture produced virus but without cellular degeneration or whether only a few cells were involved in virus propagation, the following experiment was done.† Capuchin monkey kidney cells were grown in petri

† These experiments were carried out by one of us (A.S.K.) at the California Institute of Technology through the hospitality of Drs. Renato Dulbecco and Marguerite Vogt.

TABLE II. Number of Infected Cells in Capuchin Monkey Kidney Cultures as Determined by the Plaque Technic.

Day	No. of cells in inoculum ($\times 100,000$)	No. of plaques	% of cells infected
1	2	105	.05
2	1.1	31	.03
3	1.8	27	.015
6	.1	21	.21
7	1.7	39	.023

plates according to the technic of Dulbecco and Vogt(10). These monolayer cultures were inoculated with 0.1 ml of Easton-15, 4th passage capuchin virus; the inoculum contained 2×10^5 plaque-forming particles (as determined by titration on rhesus plates). Nutrient medium was added, and at the intervals shown in Table II, the medium was removed, the plates washed several times with buffered saline, and the monolayer of cells suspended by means of 0.02% versene. The cells were sedimented and resuspended in 1 ml of nutrient medium; 0.1 ml of this cell suspension was seeded onto petri plate cultures of rhesus monkey kidney cells. After a period of 30 minutes to allow for adsorption of virus, an agar overlay was added. The plaques were counted at the end of 4 days.

The results shown in Table II demonstrate that in a population of 10^5 cells only an aver-

age of 60 cells was infected. This experiment shows that the absence of obvious cytopathic changes in the capuchin monkey kidney cultures was due to the fact that only a small proportion of cells was involved in the production of virus. The degeneration of so few cells in the culture would be very difficult to detect microscopically. The propagation of certain polioviruses in tissue cultures in the absence of observed cytopathic changes has previously been recorded(12-14), and it is likely that the present explanation of only a small proportion of cells supporting virus propagation, underlies the earlier observations.

Neurotropic properties. After 4 to 5 passages in capuchin cultures the 3 Type 1 strains were compared for the ability to grow in the central nervous system of rhesus monkeys. Attenuated strains of poliovirus have been characterized by their restricted growth in the monkey nervous system, limited chiefly to the lumbar part of the spinal cord(11). Thus an intracerebral inoculation of attenuated virus would be expected to produce disease rarely—only if the virus in some manner found its way to the lumbar region. As a corollary, intraspinal inoculation would be expected to produce paralysis of the legs, if at all. In the present experiments the viruses

TABLE III. Neurotropism of Poliovirus in Rhesus Monkeys, after Propagation without Microscopically Observed Cytopathogenicity in Capuchin Cultures.

Strain	No. of passages in TC	Route	Doses* ($\times 1000$)	Result,† day of paralysis
E-15	2	Intracerebral	8	7 (L); inc.
		Intraspinal	1.6	4 (L→A); remained well
	5	Intracerebral	20	7 (A + L); 10 (L); 14 (A + L)
		Intraspinal	4	4 (L→A); 4 (L→A); 4 (L)
NE-23	1	Intracerebral	5	8 (A + L); 8 (A)
		Intraspinal	1	4 (L); 4 (L)
	4	Intracerebral	25	14 (T + L); 14 (T + L); inc.
		Intraspinal	5	2 (L→A); 3 (L→A); 3 (L→A)
NE-34	1	Intracerebral	0.8	5 (L); inc.
		Intraspinal	0.2	4 (L); inc.
	4	Intracerebral	63	8 (A + L); 11 (L); 14 (L)
		Intraspinal	12.6	3 (L→A); 4 (L→A); 4 (L)

* Doses in inoculum determined as TCD₅₀ for rhesus kidney cultures on the day monkeys were inoculated.

† L = leg paralysis; A = arm paralysis; T = tremors; inc. = incomplete test due to non-specific death of monkey. 4 (L→A) indicates that paralysis first occurred on the 4th day, the legs being involved. Subsequently the arms (A) became involved.

propagated in capuchin kidney cells remained virulent, in the doses inoculated, after 4 or 5 serial tissue culture passages. As shown in Table III, paralytic disease was produced after intracerebral inoculation. In addition, the affinity of the virus for the CNS was demonstrated after intraspinal inoculation. When the virus was injected into the lumbar area, it multiplied there and frequently ascended the cord; this manifested itself by leg paralysis followed by arm paralysis.

Summary. Cultures of trypsinized kidneys of South American capuchin monkeys (*Cebus capucina*) supported the multiplication of Type 1 polioviruses, even though no cytopathic changes were observed by microscopic examination of the cultures. The failure to observe cytologic changes was found to be due to the small proportion of cells in the culture capable of supporting the growth of virus. After 4 to 5 tissue culture passages the viruses remained virulent for the monkey brain and spinal cord. No evidence was obtained for the propagation of Type 2 and 3

strains in the capuchin cultures.

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Measure of Irritation by Quantitative Determination of Extravasated Dye.* (22099)

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Intravenously administered colloidal dyes have been observed to concentrate at sites of inflammation(1-5). This phenomenon has been the basis for tests to measure the intensity of irritation(6-10). In such tests, visual estimations of the quantity of dye at irritated sites were graded by various systems, either direct or by comparison to standard color charts. The results of visual observations in this manner are influenced by subjective bias and are not easily reproducible.

This report describes a method for the quantitative removal of accumulated Evans blue dye from irritated tissue and its meas-

urement spectrophotometrically. This should be a reliable index of the degree of irritation, provided the amount of extravasated dye is proportional to the extent of irritation. A modification of the method of Caster, Simon, and Armstrong(11,12) for the measurement of plasma volume was used for extracting the dye from tissue.

Methods and materials. White rats, (Sprague-Dawley), 115-130 g, were anesthetized with an intraperitoneal injection of sodium pentobarbital. The skin on the posterior side of the hind legs was slit to expose the gastrocnemius muscles and 0.03 cc of the test material was injected into each muscle using a 1/4 inch, 27 gauge needle. Fifteen minutes after the injection of the test material,

* The authors wish to thank M. L. Pabst for his advice and O. S. Carpenter for advice on statistical treatments.

0.3 cc of 1% Evans blue in saline was injected intravenously in the tail vein. Thirty minutes later the animal was sacrificed, both gastrocnemius muscles removed and placed in a semi-micro Monel blender jar containing 15 cc benzalkonium chloride (Zephiran Chloride, 12.8%). The tissue and Zephiran Cl were blended at high speed in a 2-speed Waring blender for 2 minutes, 13 cc isobutyl alcohol were added, and blending was continued for 30 seconds on low speed. The mixture was transferred to a small glass-stoppered reaction tube and centrifuged at 1750 r.p.m. for 3 minutes. After centrifugation, 3 layers were visible: a top layer of isobutyl alcohol containing the dye, a middle layer of white macerated tissue, and an aqueous-Zephiran Cl bottom layer.[†] Using vacuum, the 2 bottom layers were aspirated and discarded. Approximately 3.5 g sodium sulfate anhydrous were added to the remaining isobutyl layer to remove excess water and the tube was shaken by hand for one minute. It was centrifuged at 1750 r.p.m. for 5 minutes, and the supernatant decanted into a cuvette. Absorbency readings were made at 620 m μ in a Coleman Junior spectrophotometer. By means of a standard dye curve, the absorbency readings were converted into micrograms (μ g) of dye.

Experimental. To ascertain the extent of dye recovery from the muscle tissue, the 3 layers of the first centrifugation were examined. Spectrophotometric analysis of the aqueous layer indicated that no dye was present. Microscopic examination of the macerated tissue layer showed no trace of the dye. Spectrophotometric analysis showed that the Evans blue was contained in the isobutyl layer. Therefore, this indicated quantitative recovery of the dye. The standard dye curve was prepared by processing increments of Evans blue, 10 to 150 μ g in Zephiran Cl, according to the preceding method. The curve

[†] NOTE: Occasionally, contact of Zephiran Cl with the blender assembly produced a blue color in the aqueous-Zephiran layer. A test for copper in this layer was positive. The blue color was insoluble in isobutyl alcohol, therefore, did not interfere with absorbency readings of the dye.

TABLE I. Dye Measurements for the Blank and Control Injected Rats.

Materials tested	No. of rats	Dye value, avg μ g/rat	Corrected dye value, avg μ g/rat
Blank	22	24.0	—
Saline	16	23.7	-.3
Sterile water for inj.	6	27.9	3.9

followed Beer's law confirming the work of Caster, *et al.*(13); therefore, it was used to convert the absorbency readings of all tissue extractions to μ g of dye. The amount of dye and extraneous components extracted from non-irritated muscles after intravenous injection of Evans blue was determined as a "blank." The average blank for 22 rats was equivalent to 24.0 μ g of dye. Because extraneous tissue components contributed to this value, the absorbency readings for normal muscles and test material injected muscles with dye absent were compared and found to be in agreement. Therefore, the blank was subtracted from all results obtained on the test materials to give a corrected value. Control injections of physiological saline and sterile water were tested and the results given in Table I. Physiological saline injections gave no evidence of irritation. Sterile water for injection was very slightly irritating which agrees with the findings of Weatherby(7).

Results. To test the procedure for graded responses to a known irritant, a series of concentrations of NF IX Formaldehyde were prepared and tested. The results are given in Table II. A regression line was calculated from the data in Table II and is shown in Fig. 1. Calculations of the regression line showed Slope = 27.1; Stand. Dev. = $\sqrt{107.16}$ =

TABLE II. Dye Measurements for Formaldehyde Concentrations.

Material tested	No. of rats	Corrected dye value, avg μ g/rat
Formaldehyde 1.6 %	8	43.5
.8	8	39.9
.4	18	26.3
.2	10	18.7
.1	10	6.8
.05	10	7.1

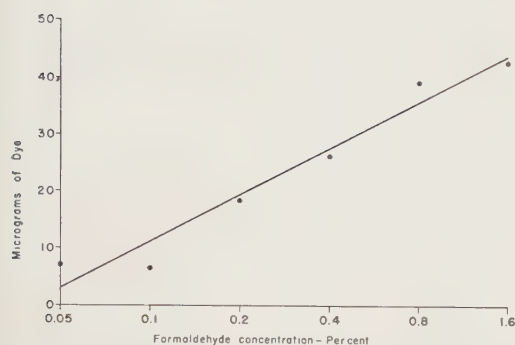


FIG. 1. Regression line for formaldehyde series.

10.352. The average dye measurements did not deviate from the regression line by more than could be attributed to experimental error. The slope of the regression line relating dye concentrations to dose concentrations was highly significant. Therefore, the method provides a quantitative measurement of degrees of irritation, assuming the degree of irritation to be proportional to the concentration of the irritant.

Preliminary examination was made of various categories of injectables to observe a range of irritation. The samples were tested

TABLE III. Dye Measurements for Injectables.

Materials tested	Concentration	No. of rats	Corrected dye value, avg $\mu\text{g}/\text{rat}$
Preservatives			
Phenol	.5 %	3	-.9
Chlorobutanol	.5	3	1.6
Methylparaben	.2	3	2.2
Propylparaben	.02	3	1.9
Vehicles			
Cottonseed oil	—	4	4.1
Peanut oil	—	4	-.6
Al. Monostearate w/peanut oil gel	2.0 %	4	-.2
Local anesthetics			
Procaine HCl	2.0 %	8	3.4
Piperocaine HCl	2.0	7	1.4
Lidocaine HCl	2.0	8	4.3
Vitamin products			
Liver inj. (erude)	2 $\mu\text{g B}_{12}/\text{cc}$	8	11.1
Idem (refined)	10	11	5.3
" " "	20	8	4.1
Nicotinic acid amide	10.0 %	4	5.6
Calcium compounds			
Ca. Gluconate	10.0 %	23	14.4
Ca. Chloride	2.5	13	35.9
Idem	1.25	10	24.0

at concentrations normally employed clinically. The results on materials examined to date are given in Table III.

The results in Table III indicate the preservatives, vehicles, and local anesthetics tested were only slightly irritating at clinical concentrations. The liver injection products did not cause irritation proportional to the amount of cyanocobalamin present; however, it appeared that refinement of the product reduced its irritating properties. Calcium chloride, which is not recommended for intramuscular injection due to its irritating properties, showed the highest degree of irritation in our study. Concentrations of 5.0 and 10.0% produced average dye values of 53.2 and 54.6 μg respectively. Because a significant increase was found in extracted extraneous components due to the irritant injection alone, these values were omitted from Table III. Dilutions of the calcium chloride from $\frac{1}{4}$ to $\frac{1}{8}$ the clinical strength showed more irritation than calcium gluconate, which also had a high order of irritation.

Because of the current interest in anti-inflammatory compounds, a preliminary study was made to determine the adaptability of this method to measure anti-inflammatory activity. The procedure was modified only in that the test animals were injected with the anti-inflammatory material previous to the injection of the irritant, Calcium Gluconate, 10.0%. Intraperitoneal injections of Solu-Cortef[†] (hydrocortisone sodium succinate), 5 mg/rat, were used to obtain the anti-inflammatory effect. The preliminary results showed that a single dose injected 1 to 5 hours before the irritant would lower the amount of dye recovered by about 25%. This indicated that the method may be modified to measure quantitatively anti-inflammatory activity.

A preliminary test of xylene treatment of the rat abdominal skin indicated that a modification of the method may be used to measure quantitatively the irritation caused by agents applied topically.

Discussion. It appears that our preliminary results cover the range from non-irritating

[†] Registered Trademark, The Upjohn Co., Kalamazoo, Mich.

to highly irritating products. After the accumulation of more data, an attempt will be made to describe irritation on a unitage basis. At present, however, it is recommended that comparisons of irritation be made within categories of compounds, since there seems to be no universal agreement on a standard irritant. The same practice would probably apply to studies of the anti-inflammatory compounds.

In the course of this investigation, alternate sites of injection and modifications of the procedure have been used. The rat ear, rat testes, rat abdominal skin, and the longitudinal back muscle of the rabbit have been used successfully as an injection site for the irritant. In each case, the procedure of dye removal was modified slightly, but essentially the method remained the same.

Summary. An objective quantitative method to measure the degree of irritation has been described. It has been used to obtain a valid dose-response curve on a known irritant, formaldehyde, and to measure the irritating properties of various categories of in-

jectables. The method has been modified to permit measurement of anti-inflammatory activity and topical irritation.

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Effect of Cyanide on Growth and Respiration in Earle's Strain L Cells.* (22100)

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Respiration in most tissues is inhibited by 3×10^{-3} M cyanide(3,8,12,14). Cyanide has been used very little as an inhibitor of cells grown *in vitro*. Danes and Leinfelder(2) noted that 0.1×10^{-4} to $.38 \times 10^{-4}$ M HCN inhibited growth of chick embryo tissue 73%. Experiments reported below were designed primarily to ascertain to what extent Earle's strain L cells, which have been grown in cul-

ture for more than 12 years(4), are cyanide sensitive.

Materials and methods. Strain L cells were obtained through the generosity of Dr. Wilton R. Earle and were cultured on glass (D-3.5 Carrel flasks) in Earle's solution consisting of 40% balanced saline, 40% horse serum and 20% chick embryo extract (in Earle's saline solution, 1:1)(4,5). The culture medium was renewed 3 times weekly and cultures gassed with 5% CO₂ in air. In experiments to test the effect of cyanide on growth, a cell suspension was obtained by gentle agitation of stock cultures. The cells were concentrated by centrifugation at 1000 rpm(7), resuspended in fresh medium after

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TABLE I. Effect of Sodium Cyanide on Growth in Earle's Strain L Cells. Growth of cells in control cultures is taken as 100.

Conc. of NaCN (molar)	No. of cultures	Avg No. of cell nuclei per culture	Growth (% of control)
1×10^{-8}	7	0	.0
1×10^{-4}	16	1.43×10^5	2.6
5×10^{-5}	9	2.19×10^5	99.6
1×10^{-5}	5	3.79×10^5	90.2
1×10^{-6}	20	1.88×10^6	95.5
1×10^{-8}	34	$1.98 \times "$	117.3
1×10^{-10}	20	$1.96 \times "$	116.2
1×10^{-12}	17	$2.21 \times "$	94.6

which 1 ml aliquots were inoculated into Carrel flasks. After 2-3 days the cultures were divided into experimental and control groups. Sodium cyanide was added directly to the medium in experimental cultures which were carried for an additional week, at which time the number of nuclei in each culture was ascertained by sample counts on a hemacytometer(13). Cells were collected for respiration experiments in the same way as for growth experiments but they were resuspended in neutralized horse serum. Neutralized horse serum contains bound bicarbonate and remains physiologically neutral when equilibrated with a CO_2 free atmosphere(16) as is employed in Warburg's direct method (15). One ml aliquots were placed in each Warburg flask. Number of cell nuclei was determined in same manner as previously indicated. Calcium cyanide was prepared and its concentration determined according to Robbie(9,10). Constant source of HCN in medium during experimentation was obtained by using $\text{Ca}(\text{CN})_2$ - $\text{Ca}(\text{OH})_2$ mixtures in center well of the Warburg flasks(11).

Results. Since number of cells used from one experiment to another was not exactly the same, determinations were made in pairs for both growth and respiration.

The results obtained on effect of NaCN on growth (Table I) indicate that Earle's strain L cells do not divide nor grow when placed in culture medium containing 10^{-3} M NaCN. After the first day in this solution all cells become detached from the floor of the flask. In 10^{-4} M NaCN the number of nuclei was scant or non-existent. Although some cells were still intact after a week it is

doubtful that growth occurred in this concentration of cyanide. In culture media containing 10^{-8} M NaCN growth is stimulated. As checked by the test for observations involving paired groups(6), the accelerated growth in 10^{-8} M NaCN was significantly different from controls ($P = \text{about } 0.01$). It appeared that growth might be stimulated in 10^{-10} M NaCN but statistically the results are not significant.

Results from experiments on effect of cyanide on respiration are presented in Table II. Oxygen uptake is inhibited 85.3% in 7×10^{-4} M HCN. It should be noted that a similar concentration of cyanide inhibited growth. Statistically, there was no evidence for stimulation of respiration with lower concentrations of HCN.

Discussion. Most of strain L cells did not grow in 10^{-4} M NaCN. In contrast, oxygen uptake was not completely inhibited in 2×10^{-4} M HCN; the mean oxygen uptake was 26.9% as much as the control. Probably difference in degree of inhibition of growth as compared to respiration can best be explained by the fact that the cells were exposed to cyanide for a longer period in the growth experiments.

It is known that the bulk of respiration is carried out by the cytochrome system. Cyanide inhibits cytochrome oxidase almost completely at concentrations of the order of 10^{-3} M, and thus inhibits about 80% of the respiration in most tissues(1).

Growth of embryonic chick heart in tissue culture is inhibited 73% in 0.1×10^{-4} to 0.38×10^{-4} M cyanide(2). The same is true for

TABLE II. Effect of Cyanide on Oxygen Consumption in Earle's Strain L Cells. Rate of oxygen consumption in control cultures is taken as 100.

Conc. of HCN (molar)	No. of determinations	Avg μl oxygen consumed/hr/10 ⁶ cells	Oxygen consumption (% of control)
7×10^{-4}	28	1.2	14.7
2×10^{-4}	28	2.0	26.9
6×10^{-5}	28	4.9	67.0
4×10^{-7}	24	7.7	109.2
2×10^{-8}	21	8.4	101.4
3×10^{-9}	28	7.5	100.4
2×10^{-10}	21	8.5	101.5
1×10^{-12}	24	8.1	105.8

other tissues. With slightly greater cyanide concentration (10^{-4} M NaCN), growth of strain L is inhibited 97.4%. Thus, although concentrations of cyanide in our experiments were slightly different from those used by other workers, the results are in agreement. Strain L is about as sensitive to cyanide as other types of tissue cells.

We noted that growth of strain L cells was stimulated with 10^{-8} M NaCN, as described by many investigators for other cells and tissues but no adequate explanation is available.

Summary. 1. Earle's strain L cells were cultured *in vitro* and the effects of different concentrations of NaCN were studied. Concentrations ranging from 10^{-3} to 10^{-12} M NaCN were tested. 2. In 10^{-4} M NaCN growth was inhibited 97.4% but in 10^{-8} M, growth was enhanced and exceeded that of controls by 17%. 3. The effect of cyanide on oxygen uptake of strain L cells in neutralized horse serum was determined by means of the Warburg Respirometer. 4. At a concentration of 7×10^{-4} M HCN, which was slightly stronger than the concentration necessary to stop growth, there was still an oxygen uptake of 14.7% as compared to controls. 5. Strain L cells possess a cyanide sensitive type of respiration similar to that found in normal cells.

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Eosinophil Response to Toxoids in Actively and Passively Immunized Mice.* (22101)

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The recent(1) development of technics for quantitatively estimating the number of cells in body cavity fluids has made it possible to determine the cellular reactions outside the blood following administration of hormones, drugs or other physiologically active substances. In a study of such reactions in the peritoneal exudate marked changes were found in the number of cells per cubic millimeter of peritoneal fluid depending upon a) the material injected, b) the degree of sensitization of the animal to that material, and c) the level of adrenal cortical hormones. An accumulation of eosinophils in the peritoneal fluid between the 2nd and 10th days appeared to be the specific local cellular response of the sensitized mouse to intraperitoneal injection of sensitizing antigen(1-3). The antigens used included pollen extract, bovine albumin, horse serum, ascaris extract and foreign erythrocytes.

This paper reports further studies concerning the local and systemic cellular responses to antigens and the chemotactic factors involved. It was found that injections of tetanus or diphtheria toxoid produced a local accumulation of eosinophils in actively immunized mice, but not in non-immunized mice or mice passively immunized with heterologous antibody.

Materials and methods. The mice used in these experiments were BBF₁ hybrids and C 57 Br/a mice from the research colonies of the R. B. Jackson Memorial Laboratory. The animals were from 2½ to 4 months of

age and both sexes were used. The mice were immunized to diphtheria toxoid (Parke Davis and Co.) by a series of 5 subcutaneous injections as follows: 0.1 ml on days 1 and 2, and 0.2 ml on days 3, 6 and 13. The final challenging injection was given intraperitoneally one week after the last immunizing injection. Two different procedures were used for immunizing mice to tetanus toxoid. The first consisted of a series of injections of fluid toxoid (Lederle) as given above for diphtheria toxoid. The second consisted of injecting subcutaneously 0.05 ml of an alum phosphate precipitated toxoid (Lederle) followed by 0.05 ml fluid toxoid 15, 22 and 29 days later. On the 36th day a challenging injection of 0.05 ml fluid toxoid was given intraperitoneally. Passive immunization was produced by injecting mice with tetanus antitoxin (Lederle) either 1 or 72 hours prior to the injection of fluid tetanus toxoid. Two different dosages were used; half the animals were injected subcutaneously and the remainder intraperitoneally.

The methods used for estimating number of blood and peritoneal cells have been published elsewhere(1,4-6). A modification of Randolph's solution(7) was found to be a satisfactory diluent for the cell determinations. Each animal was used only once for the determination of cells and then discarded.

Results. The numbers of eosinophils in the peritoneal fluids of immunized and non-immunized mice, 4 days after intraperitoneal injections of toxoid, are compared in Fig. 1. In non-immunized animals a single injection of tetanus toxoid did not increase the number significantly. The immunized animals tended to have slightly higher numbers of eosinophils in the peritoneal fluid prior to the challenging injection. The animals which received the intraperitoneal injection of toxoid, however, showed as much as a 50-fold increase in these

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IMMUNIZATION (SUBCUTANEOUS INJECTIONS)			FINAL INJECTION (INTRAPERITONEAL)		NO. OF MICE	EOSINOPHILS PER CUBIC MILLIMETER				
MATERIAL	ML. PER INJ.	NO. OF INJ.	MATERIAL	DOSE (ML.)		20,000	40,000	60,000	80,000	100,000
NONE			NONE		9					
NONE			TETANUS TOXOID	0.05	3					
TETANUS TOXOID	0.05	4	NONE		6					
TETANUS TOXOID	0.0008	5	TETANUS TOXOID	0.0008	5					
	0.002	5		0.002	3					
	0.02	5		0.02	3					
	0.05	4		0.05	6					
DIPHThERIA TOXOID	0.002	5	DIPHThERIA TOXOID	0.002	3					
	0.02	5		0.02	3					
	0.2	5		0.2	3					

FIG. 1. No. of eosinophils in peritoneal fluid 4 days following intraperitoneal injection of toxoid in immunized and non-immunized mice.

cells. Similar data were obtained with diphtheria toxoid as the antigen.

The numbers of blood leucocytes and peritoneal cells observed following an injection of 0.05 ml of fluid tetanus toxoid in actively immunized and non-immunized mice is shown in Fig. 2. In the non-immunized mice, tetanus toxoid produced no marked changes in number of blood or peritoneal cells. However, in the actively immunized mice, there was a

slight increase in blood eosinophils, and a marked increase in peritoneal eosinophils. A slight increase in the number of mononuclear cells and a transient neutrophilia also occurred.

The eosinophil responses in the peritoneal fluid on the 4th day following an intraperitoneal injection of 0.05 ml of tetanus toxoid in actively and passively immunized mice are compared in Table I. A marked eosinophilia

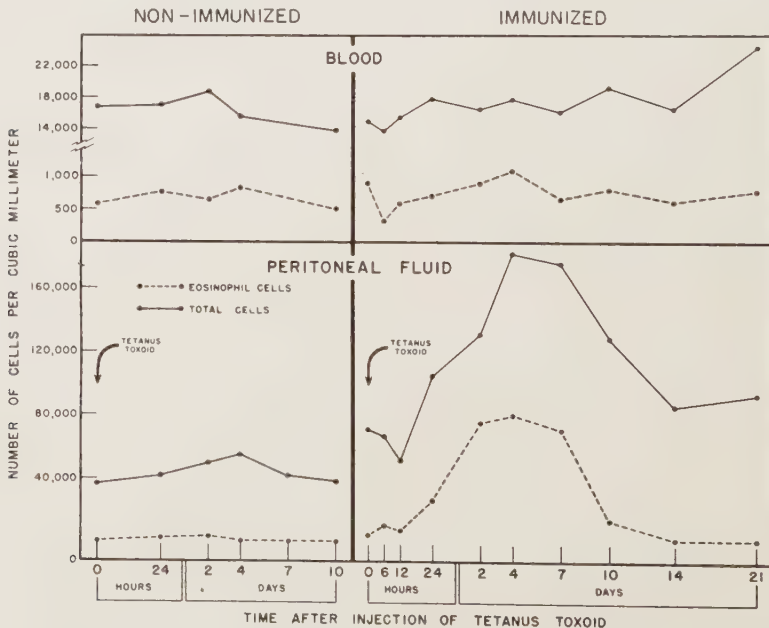


FIG. 2. Cellular responses following an intraperitoneal injection of tetanus toxoid in immunized and non-immunized mice. A total of 80 mice were used and each point represents the mean of 3 to 6 mice.

TABLE I. Comparison of Eosinophil Responses in Actively and Passively Immunized Mice 4 Days following an I.P. Injection of Tetanus Toxoid. 6 mice in each series.

Procedure for immunization				Response to tetanus toxoid eosinophils/mm ³ peritoneal fluid ± S.E.
Active	4 S. Q. inj. of 0.05 ml toxoid during 4 wk period			80660 ± 7817
Passive	A.	550 units antitoxin*	1 hr prior to toxoid†	1208 ± 166
	B.	2780	1	783 ± 271
	C.	550	72	1117 ± 127
	D.	2780	72	1158 ± 417

* Antitoxin—Lederle Globulin Tetanus Antitoxin (1 ml ≡ 5560 units).

† Half of the mice injected IP, the remainder S. Q.

occurred in every animal which was actively immunized and then reinjected with the antigen. However, there was no evidence for an increase in the number of these cells in any of the mice passively immunized with antitoxin from a heterologous species.

Discussion. These experiments demonstrate that eosinophils accumulate in the peritoneal fluid following intraperitoneal injections of either tetanus or diphtheria toxoid into mice actively immunized to the respective toxoid. This local eosinophilia did not occur in control or passively immunized animals. The number of mononuclear cells also increased following the challenging injection of toxoid but the degree of increase was less.

It is well documented that antigenic material injected for the first time is rapidly removed from the injection site. However, in the actively immunized animal the antigen becomes fixed at the site of injection. This fixation is accompanied by antigen-antibody reactions, marked stimulation of antibody production, both locally, and in the draining lymph nodes, as well as an increase in the amount of local inflammation(8-10). It is shown in this paper that these reactions are also accompanied by an accumulation of eosinophils.

In the passively immunized animal also, there is fixation of antigen, antigen-antibody reactions, and local inflammation(9,10). However, an accumulation of eosinophils does not occur in these animals. It would therefore appear that the eosinophils are related to some phase of antibody production, since by the 4th day antibody production occurs in the

actively immunized, but not in the passively immunized animal.

There has been a great deal of speculation as to which cells produce antibodies, but the eosinophil has not been considered by current workers or reviewers in this field despite earlier work suggesting this possibility(11-14). Eosinophils have been found to respond not only following the reinjection of toxoid, but also following the reinjection of a wide variety of particulate and soluble antigens, such as isoantigens, pollen extracts, albumins, horse serum, foreign red blood cells, parasitic extracts, etc.(1-3,15,16). The accumulation of these cells has been observed around the antigen whether it is introduced intraperitoneally, subcutaneously, intravenously, or via the digestive or respiratory tracts(3,17-20). In addition to the site of antigen injection, these cells also accumulate in lymph nodes draining an area of injection or infection and in the spleen following an intravenous injection of foreign protein(17,21). Furthermore, the time of local accumulation of eosinophils appears to precede slightly or to coincide with that of antibody production (22-24). It is also of interest to note that recent technics developed for staining antibody in plasma cells, also stain eosinophils in a similar manner(25,26). Finally, doses of cortisone and X-ray which inhibit antibody production also prevent the accumulation of eosinophils(27).

Summary. 1. Intraperitoneal injections of tetanus and diphtheria toxoid into actively immunized mice produce an accumulation of eosinophil cells in the peritoneal fluid which

reaches a peak at 4 days and lasts for at least 10 days. The degree of increase depends upon the dose of toxoid used. The local eosinophilia does not occur in non-immunized mice or in mice passively immunized with heterologous antibody. 2. The experiments suggest that the local eosinophilia is due neither to the antigen-antibody reaction nor to the accompanying inflammatory syndrome, but to an early stage of antibody formation during the secondary response.

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Thyroidal I¹³¹ Collection in Normal and Adrenalectomized Rats, Effect of Desoxycorticosterone Acetate and Physiological Saline.* (22102)

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Although it has often been suggested that changes in extrathyroidal distribution of iodine such as would be caused by body fluid shifts or variations in extracellular fluid volume will alter thyroidal I¹³¹ collection, little specific information on this point is available.

* Data reported are from a thesis submitted by senior author in partial fulfillment of requirements for the degree of Doctor of Philosophy, Michigan State College, 1954, and published with approval of the Director, Mich. Agric. Exp. Station as Journal Article 1810.

Boatman *et al.*(1) reported an increase in thyroidal I¹³¹ concentration ratio 3 hours after injecting I¹³¹ in rats treated chronically with desoxycorticosterone acetate (DCA). It was postulated that this may have been due to the observed increase in blood radioactivity. In contrast to this, chronic treatment of rats with DCA was reported to cause no change(2) or a decrease(3) in 24 hour thyroidal I¹³¹ collection. In man(4) a decrease was also observed. Except for the report(5) that an increased amount of NaCl in the diet

TABLE I. Effects of DCA and 0.9% NaCl on 3 Hr Thyroid Uptake of I^{131} in Adrenalectomized Rats. 10 rats in each series.

Treatment	Body wt, g	Thyroid wt, mg	Thyroid I^{131} , % dose	Urine I^{131} , % dose	Plasma I^{131} , % dose/g	Muscle I^{131} "space,"† %
Controls	161	19.4	5.6	10.0	.75	19.3
DCA, 1.25 mg/100 g	162	17.4	8.8*	9.5	.69	19.4
0.9% NaCl, 5 cc/100 g	163	19.7	7.6	22.0	.52*	24.4*
" " , 10 "	164	18.3	9.6*	19.4	.45*	24.2*

* By analysis of variance significantly different from controls at 5% level.

% dose/g muscle

† $\frac{\% \text{ dose/g muscle}}{\% \text{ dose/g plasma}} \times 89.2$.

of mice enhances the goitrogenic properties of the Remington iodine deficient diet, no reference to the effect of salt administration on thyroid function has been found. The present experiments were undertaken to determine the acute effect of DCA and physiological saline solution on short term thyroidal uptake of I^{131} . Concomitant measurements of blood plasma radioactivity and total urinary I^{131} output were made to determine the effect of the treatments on the extrathyroidal distribution of radioactive iodide.

Materials and methods. Eight to 10 days before the experiment, adult male albino rats received *ad libitum* an iodine deficient diet† with tap water to drink. The adrenals were removed at this time by a dorsal approach. The rats were given a daily subcutaneous dose of 0.25 mg DCA in corn oil. Completeness of operation was ascertained by gross examination for adrenal tissue at autopsy. Each rat received intravenously in a femoral vein exposed under light ether anesthesia 0.5 ml solution containing 20 uc I^{131} with 8.5 ug carrier I^{127} as NaI. Food and water were removed immediately before I^{131} injection. DCA, as a suspension in 0.1 to 0.2 ml corn oil and 0.9% NaCl solution (Merck Reagent), warmed to approximate body temperature, were injected intraperitoneally immediately after I^{131} . Exp. I contained 4 groups of adrenalectomized rats (Table I). The rats were placed by groups in metabolism cages for collection of urine; before removal of each rat from the metabolism cage the bladder urine was expressed manually. Rats

were sacrificed by decapitation 3 hr after administration of I^{131} , and freely flowing blood collected into a beaker containing a drop of heparin. A muscle sample was obtained from the non-injected thigh. Exp. II contained 6 groups of rats (Table II). The animals were sacrificed 1½ hr after I^{131} injection, and blood was collected as before. Plasma and urine samples were weighed to the nearest mg in 1-in. pyrex dishes. A few drops of solution containing 1% NaI, 1% casein, 1% phenol and 1% NaHSO_3 were added to each sample to inhibit oxidation and sublimation of iodine. Thyroid glands were dissected cleanly, weighed rapidly to 0.1 mg and placed in the center of similar pyrex dishes. Aliquots from the injection solution were mixed with plasma for use as standards. After drying to constant weight, all samples were counted with an end-window Geiger-Muller tube and a laboratory scaler. Counts were corrected for physical decay, and in the case of plasma, urine and muscle, for self absorption of beta radiation. Results are expressed as % of injected dose.

Results. Rats receiving both DCA and the larger dose of physiological saline accumulated significantly increased amounts of I^{131} in their thyroids at 3 hr (Table I). DCA treated rats did not differ from controls in plasma radioactivity, % of dose found in their urine or in their muscle iodide space. The increased thyroidal I^{131} accumulation occurred in saline-treated rats despite the fact that their plasma radioactivity was significantly less and their muscle iodide space and urinary excretion of I^{131} was significantly greater than in either the control or DCA-

† "Iodine Deficient Diet," General Biochemicals, Chagrin Falls, O.

TABLE II. Effects of DCA and 0.9% NaCl on 1½ Hr Thyroid Uptake of I¹³¹ in Adrenalectomized and Intact Rats.

Group	Treatment	No. of rats	Body wt, g	Thyroid wt, mg	Thyroid I ¹³¹ , % dose	Plasma I ¹³¹ , % dose/g
Adrenalectomized	Controls	21	123	12.3	.66	1.20
	DCA, 1.5 mg/100 g	20	120	11.5	.65	1.16
	0.9% NaCl, 10 ml/100 g	20	120	12.1	.95*	.91*
Intact	Controls	21	133	12.9	.46	1.11
	DCA, 1.5 mg/100 g	20	129	11.7	.45	.99
	0.9% NaCl, 10 ml/100 g	19	131	12.1	.55	.85*

* By analysis of variance significantly different from controls at 5% level.

treated groups.

The only significant intergroup difference in thyroid uptake in the second experiment (Table II) is the increase in thyroïdal I¹³¹ uptake in the saline-treated adrenalectomized group. Radioactivity of the blood plasma was reduced significantly in both the adrenalectomized and intact saline-treated rats.

Discussion. In view of the precautions taken in these experiments to standardize iodine intake, the increased thyroïdal accumulation of I¹³¹ after both DCA and physiological saline solution should represent an increased amount of inorganic iodine fixed. The early uptake of radioiodine by the thyroid is known to be influenced by the thyroid to plasma I¹³¹ ratio, by the rate of conversion of inorganic to organic iodine and perhaps by the rate of blood circulation through the gland. There is no good evidence to suggest from these data which of the 3 may have been augmented in the treated groups. In contrast to earlier observations(1) regarding the effect of chronic DCA treatment on I¹³¹ concentration ratio, the increase in 3 hr uptake of I¹³¹ after administration of DCA was observed without any increase in blood radioactivity. A most interesting situation existed in the saline treated animals in that both the 1½ and 3 hr uptake of I¹³¹ increased despite a significant decrease in plasma I¹³¹ level.

Whether it is valid to compare the effect on the thyroid of DCA and physiological saline solution cannot be shown from the data available. Yet, speculation is attractive as to whether DCA might have a typical inorganic ion or water shifting effect at the level of the thyroid itself that results in a changed capacity to concentrate iodine and that the

saline solution might have simulated the changes produced by DCA.

Summary. Adrenalectomized male rats, fed an iodine deficient diet and maintained on ¼ mg DCA daily, were injected intravenously with I¹³¹ containing 8.5 µg of iodide carrier. Thyroïdal collection of I¹³¹ increased at 3 hr after 1.25 mg DCA/100 g, but not at 1½ hr after 1½ mg DCA/100 g, the DCA being given intraperitoneally immediately after I¹³¹ in both groups. There was no change from controls in plasma I¹³¹ level or urinary I¹³¹ excretion. Under similar conditions, adrenalectomized rats showed increased thyroïdal I¹³¹ collection 3 hr and 1½ hr after intraperitoneal injection of 10 ml 0.9% NaCl/100 g. This occurred despite decreased plasma radioactivity and increased urinary I¹³¹ excretion. Intact rats studied under the same conditions showed no change from controls in thyroïdal I¹³¹ collection, but had less radioactivity in their blood plasma.

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Cortically-Evoked Pinna Movement in the Cat. (22103)

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Ferrier's(1) observation that stimulation in the cat ectosylvian region evoked ear movement, led him to postulate that this region constituted the auditory area. Bremer(2) considered that such topographic overlap of sensory and motor representations of various body parts has functional significance in indicating a mechanism subserving simple cortical reflexes. Although Ferrier's postulated auditory area has been amply confirmed by histologic and neurophysiologic studies, there are data indicating that acoustic stimulation also evokes cortical potentials in areas outside the ectosylvian gyrus. Mickle and Ades(3) and Bremer(4) observed such evoked potentials in a region roughly corresponding with somatic sensory II. We noted another region of activation more medially situated in the suprasylvian gyrus(5). Maps indicating cortical area for pinna movement reveal some discrepancies in localization (Fig. 1)(1,6,7, 8,9). Actual data are scanty; *e.g.*, Woolsey and Walzl(8) merely state that the suprasylvian area enclosed by broken lines "is the

'motor' area for the pinna" (their Fig. 10) and, similarly, Claes(9) gives no data supporting her "area for auricular movements." Ward and Clark(10) reported, as infrequent finding, that stimulation of anterior end of middle suprasylvian gyrus produced movement of the contralateral ear.

In studies of suprasylvian auditory response area our observations indicate a sensory-motor overlap, which reconcile, to some degree, discrepant findings in earlier reports.

Methods. Observations were made on 10 adult cats anesthetized with Dial-urethane, 50 mg/kg, or Nembutal, 35-50 mg/kg intraperitoneally, and on 2 modified "encéphale isolé" preparations. In the latter, operative procedures were carried out under ether anesthesia and the animal allowed to regain consciousness under artificial respiration after transection of the cord at C3 and infiltration of operative sites with procaine. The C3 level, rather than the bulbospinal level of Bremer, was chosen since a major portion of innervation of the pinna is derived from C1

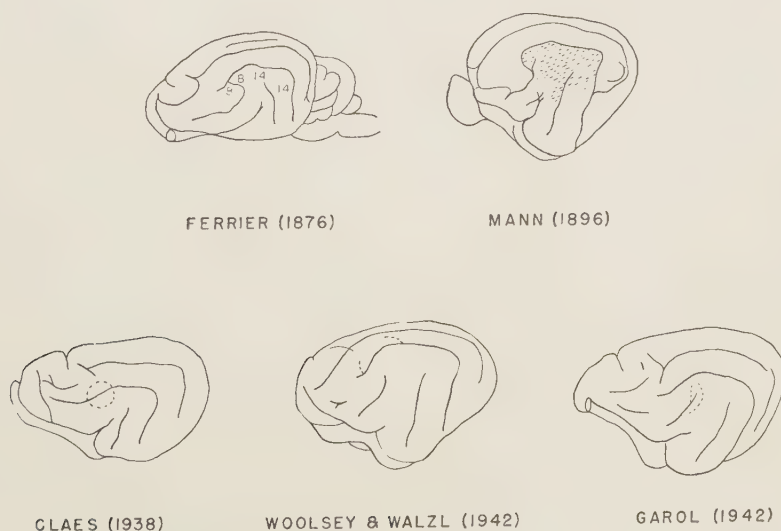


FIG. 1. "Motor area" for pinna movement as reported by other authors (reproductions of original diagrams).

and C2 segments. Various degrees of exposure of one or both hemispheres were made and stimulation carried out with bipolar platinum electrodes (inter-electrode distance 0.5-1 mm) leading from a Grass square wave stimulator. Optimal parameters of stimulation were determined and observations of pinna movements were recorded cinematographically. In some experiments in which the areas of acoustically-evoked potentials were defined, the electrocorticogram was recorded by a Grass Model III electroencephalograph, using wick electrodes.

Results. Exploration of lateral surface of cortex, made accessible by wide exposure, revealed that pinna movements were evoked from 2 small discrete areas, one in the ectosylvian gyrus (ES), lying within Auditory I area, and the other in the suprasylvian gyrus (SS) roughly corresponding with suprasylvian auditory receptive area mentioned above (Fig. 2). Since wide exposure of a hemisphere virtually destroys the ipsilateral pinna, smaller exposures were made and care taken to preserve muscle and fascial attachments of the pinna as much as possible. It was then noted that the 2 responses differed significantly *i.e.*, ES response was always bilateral, whereas SS response was always unilateral, involving the contralateral pinna.

Actual movement of the ear varied somewhat from animal to animal, the most consistent pattern from SS stimulation consisting of an initial pricking up and cupping of the contralateral ear. If the stimulus was continued, this was followed by a "laying back" of the ear; *i.e.*, ear was retracted so that it lay in the plane of the dorsal convexity of the

skull. With ES stimulation, the most common response was a pricking up of both ears, without cupping.

Both areas were approximately equal in size, and under conditions of these experiments covered approximately 20-30 sq mm in a given animal. No consistent differences in pattern of pinna movement could be determined by stimulation of different points within the responsive areas, even with closely spaced electrodes and threshold stimulus intensities.

The most effective parameters of stimulation for SS were 40-60/sec, pulse duration 5-10 msec, and 4-6 volts output. Pulse durations briefer than 2 msec rarely evoked responses. The required duration of stimulus was a few seconds. Changing parameters of frequency and pulse duration produced no significant changes in pattern of ear movement, if the stimuli were effective at all. With suprathreshold stimulation the required stimulus duration decreased, and initial pricking up of ears tended to disappear, movement consisting mostly of retraction. Antecedent stimulation frequently facilitated the responses from SS, so that with a series of stimulations, latency might be shortened to a small fraction of a second. In this way, a previously ineffective stimulus frequency of 5 or 10/sec might now become effective and evoke pinna twitches which followed the stimulus frequency. No marked extinction phenomena were noted.

ES required similar parameters of frequency and pulse duration, but in general had somewhat higher threshold than SS. In a number of experiments with good SS responses, no pinna movements could be elicited from any portion of the ectosylvian gyrus. The converse was not observed. When present, ES responses required somewhat higher voltages (6-10 v) and longer stimulus durations. No gross facilitation by antecedent stimulation was demonstrated for ES responses, which were also more susceptible to anesthesia, disappearing earlier than SS with increasing depth of narcosis.

Prolonged stimulation of SS frequently resulted in after-discharge manifested by



FIG. 2. Cortical areas from which pinna movements are evoked by electrical stimulation.

twitches of contralateral pinna. These seizures sometimes spread to involve contralateral face, but frequently remained limited to the pinna. Such post-stimulus effects were never observed after ES stimulation, despite use of strong and prolonged stimulation. Local application of strychnine to SS was followed by spontaneous twitches of contralateral ear, twitches being coincident with strychnine spikes as recorded electrocorticographically. Although less consistently obtained, strychninization of ES evoked bilateral ear twitches of lesser magnitude.

Ablation of ectosylvian or cruciate gyri did not influence responses obtained from SS stimulation, nor did ablation of SS abolish responses evoked by stimulation of ES.

SS responses were easily obtained from the modified *encéphale isolé* preparations, the fundamental pattern being similar to that observed in anesthetized animals.

Frequently, but not consistently, stimulation of SS evoked conjugate deviation of eyes to the opposite side, in addition to contralateral pinna movement. Such eye movements were not observed with ES stimulation, although reported to occur by Ferrier.

Discussion. There are 15 muscles which move the external ear of the cat; *i.e.*, change its orientation in space, and an additional 6 which change orientation of its parts to each other; *e.g.*, "cupping"(11). With such a complex of muscles, a considerable range of variability of movement pattern is conceivable. However, movements which have meaning to the human observer are limited to a few gross patterns such as pricking up of ears, as though "alerted," and laying back of ears, as though enraged. Behavioral interpretations of observed ear movements evoked by electrical stimulation face obvious difficulties. Nevertheless, the demonstration of unilateral responses from one cortical region as opposed to bilateral responses from another, represent two significantly different patterns of motor response.

It is difficult to determine, from reports of other investigators, whether any differentiation between bilateral and contralateral pinna movements has been made. It appears that

other observers considered that ear movements represented an exploratory or alerting reaction to sound. Behaviorally, at least two types of auditory alerting must be considered. In one there is a generalized alerting; *i.e.*, both ears prick up and the animal becomes generally attentive without specific reference to the source of stimulus. This seems a response mediated by the ES area. In the other type, the animal becomes attentive to the particular region in space from which acoustic stimulus arises. One or both ears may respond, with the ear ipsilateral to the stimulus source turning laterally or cupping, and head and eyes perhaps turning to that side.

Our studies of suprasylvian potentials evoked by acoustic stimulation indicate that these responses are largely evoked by stimulation of contralateral ear. Since this same area, when stimulated electrically, evokes pinna movement restricted to the opposite ear and, frequently, conjugate deviation of eyes to opposite side, it is suggested that this suprasylvian area mediates a cortical reflex concerned with "directed alerting"; *i.e.*, with an alerting reaction focussed toward the side from which acoustic stimulus originates.

Only part of the cortical efferent discharge evoked by afferent volley is directed towards motoneurons of brain stem or spinal cord, and a study of sensory-motor overlap gives information on only one aspect of function of such a region. Ability to localize sound in space has been attributed to asymmetrical activation of ectosylvian auditory area of the two hemispheres(12). The data here presented suggest that the suprasylvian region may also play a role in the mechanism of sound localization.

Summary. 1. Pinna movements in the cat are evoked by electrical stimulation of two discrete cortical areas. One, in the ectosylvian gyrus (ES) evokes movements of both ears; the other in the anterior suprasylvian area (SS) is of lower threshold, and evokes movement of the contralateral ear alone. 2. Both areas are topographically situated in regions in which acoustic stimulation evokes electrical responses. 3. The 2 areas of sen-

sory-motor overlap are considered to mediate 2 different cortical reflexes, the ES response of generalized alerting to unlocalized sound, and SS response one of oriented alerting to localized sound.

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Mechanical Fragility of Human Neutrophilic Erythrophagocytes. (22104)

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It has been suggested that the erythrophagocyte may be a fragile cell(1) but controlled studies have not appeared in the literature. Studies presented here were designed to obtain quantitative data on this point as part of a study of properties of the erythrophagocyte and the nature of erythrophagocytosis. Erythrophagocytosis can be produced *in vitro*(2) by incubating phagocytic leucocytes with red cells sensitized by antibodies or other surface altering substances(3,4). A method for producing erythrophagocytes in high percentages in a uniform manner using iso-agglutinins was developed and used.

Definitions and method. For purposes of this experiment the erythrophagocyte is defined as any white blood cell which has ingested one or more red cells, red cell ghosts, or large fragments of either. Finely vacuolated cytoplasm in a leucocyte was not considered evidence for erythrophagia. White cells found erythrophagocytic under suitable conditions included neutrophiles, eosinophiles, and monocytes. This is in agreement with previous work(5,6). Of this group of cells only the neutrophile was present in appreciable percentages in the blood used, hence, our

data applies to the neutrophilic erythrophagocyte. Cell survival time refers to *in vitro* period of morphologic integrity as indicated by presence of cell membrane or well defined cytoplasmic border seen by phase microscopy, chamber examination using special diluent, or on differential smear. Blood from normal, type A and O Rh negative white adult donors was used as source of red cells and anti-sera respectively. In plasma from type O donor, the anti-A titer was at least 1:512. The same red cell and anti-sera donors were used throughout. Anti-sera showed no agglutinating or lysing properties against the leucocytes used. White blood cells were obtained from normal type O adults and adult patients with elevated leucocyte counts and high neutrophile percentages. Blood was drawn in 5 cc samples to which 0.01 cc heparin (50 mg/cc) had been added. Erythrophagocytic activity does not appear to be altered by presence of anticoagulants (4,5). Blood from each donor was centrifuged 3 minutes at 2500 rpm promptly after drawing. The buffy coat from the leucocyte donor was removed with red cells and plasma to yield a mixture containing approximately 60% cells and 40% plasma by volume. Cells

TABLE I. Results of 14 Determinations in Normal Adults of 50% Survival Times (ST50) of Control and Experimental Neutrophils Subject to Mechanical Trauma.

ST50 experimental	ST50 control	Ratio of ST50 control to ST50 exper.	% erythro-phagocytes in total WBC	% neutrophiles erythrophagocytic	Total WBC
135	265	1.96	40	97	6850
144	289	2.01	48	100	6950
149	285	1.91	56	100	7400
135	255	1.89	60	99	6700
122	252	2.07	52	98	8050
108	250	2.31	66	96	7250
132	268	2.03	48	100	7250
144	295	2.05	58	100	8500
180	378	2.10	56	96	7650
180	360	2.00	68	99	6200
333	386	1.16	55	98	6950
144	351	2.44	64	100	7900
135	345	2.56	48	100	6950
117	304	2.60	56	96	6100

in this mixture were transferred to Wintrobe tubes placed in 37°C water bath, and tilted for rapid sedimentation by gravity to produce a leucocyte rich plasma. Final incubation mixtures placed in 5 cc test tubes consisted of 1.5 cc of anti-A plasma to which had been added 0.50 cc of leucocyte rich anti-A plasma and 0.025 cc of saline washed type A red cells. The leucocyte count in these mixtures varied from 8000-10000 cells/cu mm. When necessary, in samples from white cell donors with marked leucocytosis adjustment of cell count to this level was made by dilution with plasma from the same donor before adding the samples to incubation mixture. Mixtures were incubated 1 hour in 37°C water bath then transferred to 125 cc Erlenmeyer flasks, each containing six 3 mm round, glass beads, and placed on mechanical rotator at 180 rpm. The initial aliquot was taken prior to start of mechanical trauma. Temperature during period of trauma was maintained at approximately 35-37°C by placing flasks in a closed, insulated, electrically warmed box fastened to mechanical rotator. White cell counts and differential smears were obtained on initial sample and on each 0.15 cc interval aliquot. 300 consecutive white cells were counted for each differential. No attempt was made to differentiate between young and old neutrophilic forms of erythrophagocytes. Two separate pipettes and chambers were used for each cell count. All cells were counted using high power objective. A fresh solution of

Giemsa stain in isotonic saline was employed as diluent. Turk's solution containing acetic acid or any hypotonic diluent tended to rupture erythrophagocytes. Our method left some red cells intact but their relatively small numbers did not interfere with leucocyte counts. Some clumping of white cells occurred after trauma began, as observed by others(7) but reproducible white counts were obtainable. Clumping was frequently marked if white cells other than those from type O donors were used. Comparison of differential counts made on smears with those made in a phase counting chamber using phase microscopy revealed close agreement showing that smearing did not measurably destroy erythrophagocytes. This was supported less directly by relatively slight decline in erythrophagocytic percentage in untraumatized control groups examined by smear. Control mixtures were employed with each series. The major control group contained anti-A plasma and white blood cells in the same quantity as in experimental group, however, type O red cells were substituted for type A to prevent their sensitization and subsequent ingestion by white cells. Other mixtures identical with those mentioned above but not subject to mechanical trauma were also used. In each instance, cells and plasma were obtained from the same donors as in experimental series.

Results. The results of 14 determinations using white blood cells from normal adult type O donors are shown in Table I. Total

white blood count of this group varied from 6100 to 8500 leucocytes/cu mm. In each case the number of stab and juvenile forms was less than 6%. Neutrophilic erythrophagocytes contained an average of over 2 ingested red cells, ghosts, or large fragments and at times as many as 9. About $\frac{3}{4}$ of these appeared to be ghosts. Following a Lepehne (8) stain for hemoglobin varying amounts of this pigment could be demonstrated in many red cell ghosts. Donor No. 1 provided antisera for the entire group as well as white cells for the first determination. Survival times of 50% of cells (ST50) were obtained from curves plotting percentage of remaining cells against time. In every instance ST50 of the control neutrophils exceeded that of erythrophagocytic neutrophils. To provide a measurement of relative fragility of cells under those experimental conditions the ratio of ST50 of the control to ST50 of experimental groups was tabulated (column 3, Table I). The average value of this ratio equaled 2.08 with standard deviation of 0.34.

In patients with leucocytosis and neutrophilia with varying degrees of "shift to the left" similar survival times were done. These are shown in Table II. The ratios for relative fragilities in this group were somewhat higher than for normals but of the same general order of magnitude.

The disappearance of experimental neutrophilic erythrophagocytes and control neutrophils from 14 normal donors are shown in Fig. 1, based on average values obtained from determinations of absolute neutrophilic erythrophagocyte and neutrophile numbers derived by multiplying total white cell count of an aliquot with percentage of these cells in the differential smear of the same aliquot. Significant differences in disappearance of neutrophilic erythrophagocytes and neutrophils were not found in those systems which did not receive mechanical trauma.

Discussion. Results indicate that within the artificial system employed, neutrophilic erythrophagocyte is about 2 times more fragile than neutrophile which has not ingested red cells or red cell ghosts. This means that antibody which is active against

TABLE II. 50% Survival Times (ST50) of Experimental and Control Neutrophils Subject to Mechanical Trauma in 6 Patients with Leucocytosis and Neutrophilia.

ST50 experimental	ST50 control	Ratio of ST50 control to ST50 exper.	% erythrophagocytes in total WBC	% neutrophils phagocytic	% juveniles and stabs in neutrophile group	Total WBC ($\times 1000$)	Disease
117	380	3.26	92	99	42	24.5	Bronchiectasis
104	348	3.34	90	100	36	18.5	Cerebral hemorrhage & bronchopneumonia
108	290	2.69	90	98	50	16.5	Pyelonephritis
126	370	2.94	86	97	36	21.0	Carcinoma of cervix with metastasis
147	384	2.62	88	100	64	28.0	Carcinoma of prostate and bronchopneumonia
117	450	3.84	88	98	32	14.5	Cirrhosis of liver

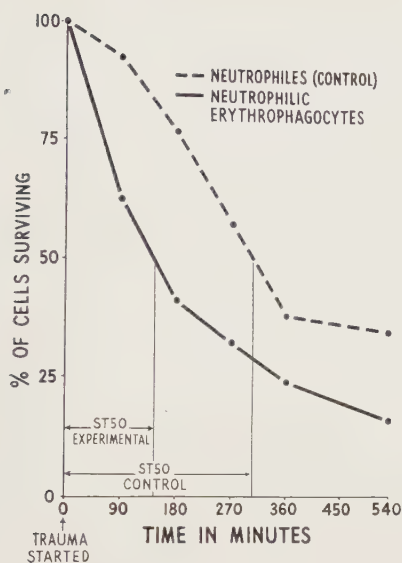


FIG. 1. Disappearance of neutrophilic erythrophagocytes and control neutrophils subject to mechanical trauma with comparison of 50% survival times.

red blood cells can shorten survival time of neutrophils present in the same system by promoting their conversion to fragile erythrophagocytic forms. This occurs in the absence of anti-leucocytic activity in the antibody. An exact measurement of this enhanced fragility, under these special circumstances, may be less important than the fact that fragility appreciably increases. Jordan (9) has summarized the theories relating frequent association of leucopenia and especially neutropenia with erythrophagocytosis *in vivo*. There are two main concepts: (1) entrapment of large erythrophagocyte in capillaries of the lung, liver, and spleen and (2) action of leucocytic lysins. It is suggested from our results that leucopenia from enhanced neutrophile fragility should be investigated as a possible explanation of this association.

Further, this enhanced fragility may help explain other findings associated with some acquired hemolytic anemias. It has been observed that frequently erythrophagocytosis

is absent or minimal in films of peripheral blood in cases which have demonstrable red cell sensitization (positive direct anti-globulin test). However, if blood from the same sources is incubated *in vitro* at 37°C, large numbers of erythrophagocytes may be produced (1), suggesting that removal of circulatory trauma allows these mechanically fragile forms to survive, although other explanations can be given. While considerable emphasis previously has been on the role of phagocytes in reducing the number of circulating red blood cells, this study gives evidence for the importance also of the reverse consideration, namely, possible role of the red cell in reducing the number of circulating neutrophilic phagocytes.

Summary. Neutrophilic erythrophagocytes were produced in high percentages by sensitizing type A human red cells with anti-A isoagglutinin in the presence of viable leucocytes. These erythrophagocytes were subject to mechanical trauma and found appreciably more fragile than neutrophils which had not ingested red cells but which were treated in an identical manner. The possible significance of this decreased survival time is briefly discussed.

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Dihydrostreptomycin Inhibition of Cysteine Desulphydrase in Cell Free Extracts of *Escherichia coli*. (22105)

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The inhibitory effect of streptomycin on hydrogen sulfide production from organic and inorganic sulfur compounds by non-proliferating *Brucellae* and *Enterobacteriaceae* was reported by Olitzki(1,2). It was shown that preincubation of bacteria with streptomycin inactivates enzymes responsible for H₂S formation from various organic and inorganic sulfur compounds. These experiments were carried out using whole bacteria. The present paper examines the action of streptomycin on cysteine desulphydrase in cell free extracts of *E. coli*.

Materials and methods. The organism used was *E. coli* B/r strain. Cysteine was determined by the method of Binkley and Nakamura(3). Streptomycin was assayed by the method of Sulitzeanu and Olitzki(4). This method is based on growth intensity of a streptomycin dependent strain of *Vibrio* and the inhibition by streptomycin of a sensitive one. Sulfide was determined by the method of Delwiche(5). Preliminary experiments having shown that cysteine desulphydrase of *E. coli* B/r is an adaptive enzyme, the bacteria were grown on the surface of "Difco" nutrient agar in Roux bottles, to which cysteine at a final concentration of 0.1% was added aseptically. After 18 hours incubation at 37°, the cells were harvested in distilled water, centrifuged, twice washed in phosphate buffer (M/15, pH 7.2), disrupted by sonic vibration in a Raytheon Magnetostriction Oscillator for 15-20 minutes and centrifuged at 10000 rpm for 10 minutes. The supernatant solution was used in all experiments. Protein content of the extracts was determined by the biuret method in a Coleman Jr. spectrophotometer at 540 m μ , using a standard curve prepared for this purpose. Preliminary experiments on dihydrostreptomycin inhibition of H₂S production by cell free extracts of *E. coli* had shown a marked correlation between the degree of in-

hibition and the time of preincubation of streptomycin with the extracts before the addition of substrate. Preincubation of extracts with streptomycin for 15, 30, 60 and 90 minutes resulted in about 5, 13, 30 and 50% inhibition respectively. Preincubation of extracts with streptomycin for 2 and more hours caused almost complete inhibition of H₂S production. In all experiments reported in this paper the extracts were therefore preincubated at 37° for 2 hours with streptomycin and cysteine was then added. In order to ascertain that the loss of hydrogen sulfide production was not due to enzyme inactivation merely by incubation, in each experiment controls without streptomycin were subjected to the same incubation. All experiments were carried out in small test tubes closed by rubber stopper to prevent loss of hydrogen sulfide. For sulfide determination the reaction was stopped by the addition of sodium hydroxide. For cysteine determination the reaction was stopped by trichloroacetic acid. In some experiments the extracts after preincubation with streptomycin were dialysed against distilled water. The dialysis was carried out in a cold room at 5°, with stirring for 18 and 35 hours before the substrate was added, the water being changed every 8 hours.

Results. *Effect of streptomycin concentration on H₂S production from cysteine.* To determine the minimal concentration of streptomycin required for almost complete inhibition of hydrogen sulfide production, 1 ml of extracts, equivalent to 7 mg protein, was preincubated for 2 hours with amounts of streptomycin ranging from 40 to 0.3 μ g. After preincubation 10 μ moles cysteine was added. The pH of the reaction mixture was adjusted by phosphate buffer to 7.2 and sulfide was determined after 2 hours incubation at 37°. Samples without streptomycin were run as controls. The results (Table I)

TABLE I. Effect of Streptomycin Concentration on H_2S Production from Cysteine.

Amt of streptomycin (preincubated $\mu g/7$ mg protein)	Sulfide produced ($\mu moles$)	% inhibition
40	.19	92
20	.19	92
10	.31	87
5	.70	71
2.5	1.4	42
1.25	1.58	35
.6	1.72	29
.3	1.82	25
0	2.43	0

Conditions as described in text.

show that the lowest concentration of streptomycin required for more than 90% inhibition of hydrogen sulfide production from cysteine was 20 μg per 7 mg protein.

Effect of cysteine concentration on H_2S production in presence and absence of streptomycin. Increasing amounts of cysteine were added to the extracts with and without preincubation with streptomycin in order to determine whether or not the inhibition of hydrogen sulfide production by streptomycin is of a competitive type. The results are summarized in Table II. Increasing the cysteine concentration until the system was saturated with the substrate caused a corresponding increase in hydrogen sulfide production by extracts not preincubated with streptomycin. The failure to produce hydrogen sulfide by preincubated extracts was not overcome by increase of cysteine concentration, indicating that the inhibition of hydrogen sulfide production from cysteine by streptomycin is not of the competitive type.

TABLE II. Effect of Cysteine Concentration on H_2S Production by Extracts of *E. coli* with and without Streptomycin.

Cysteine ($\mu moles$)	Sulfide produced ($\mu moles$)	
	Without streptomycin	With streptomycin
10	4.10	.30
20	7.18	.30
40	8.12	.30
80	5.93	.26

Each vessel contained: Phosphate buffer (pH 7.2) 100 $\mu moles$, cysteine as indicated, extract equivalent to 7 mg protein, streptomycin (when added) 20 μg . Sulfide was determined after 3 hr incubation at 37°.

To exclude the possibility that the loss of hydrogen sulfide production was due to some interaction between dihydrostreptomycin and cysteine the following experiments were carried out:

1. To extracts with and without streptomycin, cysteine was added and after 3 hours of incubation at 37° aliquots were tested for the presence of cysteine. The results showed that in the presence of streptomycin all added cysteine remained free and could be recovered. The following experiments with dialysed extracts served the same purpose.

2. Extracts without and in the presence of streptomycin (20 $\mu g/7$ mg protein) were dialysed as described above. After dialysis,

TABLE III. Effect of Dialysis of Extracts of *E. coli* on H_2S Production from Cysteine, with and without Preincubation with Streptomycin.

Previous treatment of extracts		Incubation with cysteine (hr)	Sulfide produced ($\mu moles$)
Preincubation with streptomycin	Dialysis (hr)		
Not preincubated		1	1.25
" "		2	2.12
Preincubated		1	.12
" "		2	.15
Not preincubated	35	1	.63
" "	"	2	1.20
" "	"	3	1.79
Preincubated	"	1	.07
" "	"	2	.10
" "	"	3	.10

Each vessel contained: Extract equivalent to 7 mg protein, cysteine 10 $\mu moles$, phosphate buffer (pH 7.2) 100 $\mu moles$, streptomycin (when present) 20 μg . Final vol, 3 ml.

cysteine was added and hydrogen sulfide determined after various periods of incubation. Dialysis for 35 hours caused about 40% loss of activity of cysteine desulfhydrase, in extracts without streptomycin. An almost complete loss of hydrogen sulfide production by preincubated extracts was observed when more than 75% of the original amount of streptomycin was removed by dialysis as shown in Table III. It was observed that after 18 hours of dialysis of an aqueous solution of streptomycin containing 125 μg in a volume of 5 ml, 5.5 $\mu g/1$ ml remained and after 35 hours of dialysis no streptomycin was left. When extracts con-

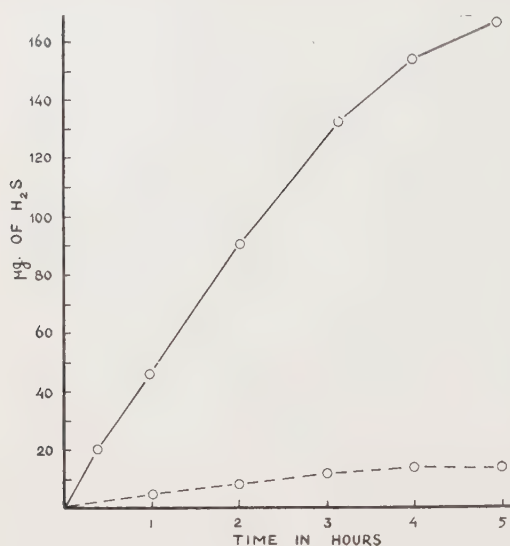


FIG. 1. The kinetics of H_2S production from cysteine by extracts of *E. coli* without and after preincubation with streptomycin. Each vessel contained: Extracts equivalent to 7 mg protein, cysteine 10 μ moles, phosphate buffer (pH 7.2) 100 μ moles. Final vol, 3 ml. Continuous line (—) H_2S production by extracts without streptomycin. Dotted line (----) *Idem*, with streptomycin (20 μ g).

taining the same concentration of streptomycin (25 μ g/1 ml extract equivalent to 10 mg protein) in the same total volume were dialysed under the same conditions, the amount of streptomycin left was 19.8 and 7.1 μ g/1 ml respectively. These experiments showed that in the presence of extracts of *E. coli*, diffusion of streptomycin was retarded. However after 35 hours of dialysis the concentration of streptomycin was too low to give appreciable inhibition of hydrogen sulfide production from cysteine by non dialysed extracts. In spite of this there was almost complete inhibition of hydrogen sulfide production by dialysed extracts, indicating the irreversibility of cysteine desulfhydrase inhibition by streptomycin.

Discussion. It seems evident that low concentrations of streptomycin (20 μ g/7 mg protein) caused almost complete and irreversible

inactivation of cysteine desulfhydrase of *E. coli*. The inactivation became apparent after 2 hours preincubation of streptomycin with extracts. A shorter preincubation time caused a corresponding smaller effect. Similar observations were made by following authors: Geiger(6) observed that in *E. coli* streptomycin inhibited aspartate oxidation but only after 1-2 hours had elapsed. Umbreit and Tonhazy(7) had shown that streptomycin inhibition of oxygen uptake by rat kidney mitochondria in presence of fumarate and pyruvate was evident only when streptomycin has been in contact with mitochondria for a period of 2 hours in the cold before the substrates were added. Paine and Clark(8) had shown that the inhibitory effect of streptomycin on oxygen uptake by resting cells of *E. coli* became apparent after 1-2 hours incubation.

Summary. 1) The effect of streptomycin on the cysteine desulfhydrase in cell free extracts of *E. coli* is reported. 2) 2.5 μ g of streptomycin/1 mg of crude extract protein caused almost complete inhibition of enzyme activity. 3) Inhibition is of a non-competitive type, since it is not reversed by increasing concentrations of substrate. 4) Inhibition is irreversible, since the removal of streptomycin by prolonged dialysis did not cause a restoration of enzyme activity.

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Rapid Freezing and Thawing of Whole Blood. (22106)

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Destruction of erythrocytes following freezing and thawing is so familiar a phenomenon as to have become a recognized procedure for obtaining complete red cell hemolysis. Only in very recent years have the basic principles underlying freezing in biology been sufficiently well established to suggest some of the causes of this hemolysis as well as means by which it might be avoided. It has long been recognized that there is a substantial difference between the results of rapid and slow freezing in biology. More recently this has been placed on a less empirical basis by the demonstration that slow freezing causes the formation of exclusively extracellular ice crystals which plasmolyze but do not necessarily rupture the cells while rapid freezing causes the formation of numerous intracellular ice crystals(1). When blood is slowly frozen, it has been shown that it is the dehydration and resultant electrolyte concentration which destroys the cell rather than the physical presence of the extracellular ice crystals(2). The success of glycerine in preventing the hemolysis of frozen erythrocytes can be attributed to the reduction in dehydration effected by the ability of glycerine to bind to itself and retain in solution much of the water which would otherwise have crystallized(3). Rapid freezing entails a considerably different problem and the only hope for subsequent survival lies in obtaining a crystal size sufficiently small to be benign despite the intracellular distribution. Luyet(4) has recovered morphologically intact erythrocytes after rapidly freezing and thawing a thin smear of blood on a cover slip but there are no other reports known to the authors in which the surface-to-volume limitations for rapid heat exchange were adequately recognized. Where the formation of extremely small intracellular crystals is tolerated it is presumed that

the denaturing effect of removing water from solution can be reduced to an insignificant level by the rapid attainment of and storage at low temperature.

Since both the attainment of small ice crystals and the prevention of denaturation depend upon very rapid freezing to low temperature, the major problem is simply one of efficient heat exchange. Studies in the kinetics of freezing and thawing(5) have shown that the most efficient shape for heat exchange is the very small sphere and that the exchange of latent heat, particularly in thawing, is so demanding that every possible advantage that may be gained through specimen geometry must be exploited to the utmost if the transition from liquid to solid and back to liquid is to be sufficiently rapid. Further discussion of the theory and mechanism of the freezing of whole blood may be found in a more extensive paper on this subject to be published elsewhere(6).

Method. In view of the above remarks it can be seen that the first obstacle to successful rapid freezing of whole blood is the attainment of sufficiently fine droplet form without injury. Many means have been tried: the conventional atomizer, spinning disc and many types of jet, all with widely varying degrees of success. The method ultimately adopted was to spray the blood through a rapidly oscillating capillary jet drawn to an 0.18 mm aperture from 2.5 mm I.D. vinyl tubing. The fine droplets, ranging from .45 to .9 mm in diameter, are sprayed directly onto the surface of liquid nitrogen or liquid air where they float for a second, then sink to the bottom of the vessel. Reconstitution is achieved simply by sprinkling the frozen blood, which now resembles a fine sand, into plasma or physiological saline at 42°C. Throughout the developmental stages, spontaneous hemolysis was used as the criterion for erythrocyte injury.

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TABLE I. Post-Transfusion Survival of Erythrocytes after Freezing and Thawing Expressed as Percent of Whole Blood Radioactivity 15 Min. after Injection.

Subject	Days post-transfusion															
	1/4	1	2	3	4	5	6	7	8	12	13	20	31	37	44	56
HM (autog.)	94	86	82	77	87		70	54			55	45	32	35	21	15
EK (homol.)	101	82	78	67	69	64			52	55		01				12

Results. Following the standardization of the jet method as the least traumatic, recovery of intact erythrocytes was still rarely better than 50%. Because of these consistently poor although promising results, a compromise was introduced in the form of added dextrose designed to reduce the crystal growth rate(6) as well as to bind additional water and reduce the ultimate amount of ice formation. This produced an immediate improvement in percent recovery when added at the rate of 0.07 g/cc whole blood, reducing the spontaneous hemolysis to between 15 and 20%.

At this stage, 100 ml of dog blood were frozen, thawed, tagged with Cr⁵¹(7) and reinfused into 2 dogs in order to determine the *in vivo* survival of the erythrocytes. Partly because of the fragility of dog erythrocytes and partly because of poor blood handling technic in this particular experiment, over 60% spontaneous hemolysis resulted from freezing and thawing alone. Of those cells remaining intact, only 18% were still present in the recipient circulation after 24 hours.

Following this equivocal result, every step of the procedure was reexamined. No major changes were made, but many minor improvements such as a change from polyethylene to vinyl tubing, covering the tubing with a rubber coat where it passed under the rollers of the peristaltic pump, making tubing and jet one piece instead of replaceable through a Luer-lok joint, and increasing the efficiency of heat exchange in the thawing bath, all contrived to reduce the damage to freshly drawn, citrated blood with added dextrose to consistently less than 3% spontaneous hemolysis after freezing and thawing. When dextrose was not added, 10 to 15% hemolysis could be expected. Inasmuch as another dog transfusion, whether positive or negative, would have to be confirmed in man, this intermediate step

was bypassed and a reinfusion of Cr⁵¹ tagged blood was made in a human volunteer.

Two 100 ml aliquots of blood were drawn, each into a solution containing 0.32 g sodium citrate, 0.1 g citric acid and 7.3 g dextrose in 25 ml of solution. One of these contained, in addition, 1.0 μ c of Cr⁵¹ as sodium chromate in 2 ml of solution. The tagged aliquot was frozen by the standard technic described above under experimental procedure. Fifty ml of plasma were removed from the untagged aliquot and used to cover the bottom of the reconstitution vessel. Because of the unaccustomed attempt to maintain a semblance of sterile technic, portions of the thawing procedure were clumsily handled and 6% hemolysis resulted. An aliquot of this blood containing an estimated 10 ml of cells was reinfused into the original donor by intravenous drip. Other than a brief psychosomatic response, no adverse clinical reaction was observed. Samples withdrawn 5, 10 and 15 minutes following infusion showed no change in radioactivity. A normal red cell volume calculated from these values indicated no immediate loss of cells after injection. At 6 hours a fall of 6% was observed. Subsequent samples taken daily for a week and at weekly intervals thereafter showed a slightly accelerated biological decay during the first 2 weeks with normal survival thereafter. (Table I). A second transfusion, this time homologous, is also charted in Table I. Mechanical failure again dogged the thawing apparatus and was presumably responsible for much of the 7% spontaneous hemolysis. Subsequent biological decay was again accelerated. Between the second and third week there was virtually a complete loss of blood radioactivity. A battery of tests failed to demonstrate any antigen in the donor's erythrocytes which might have been responsible. Preliminary experiments in rabbits indicate

that there may be an alteration in the attachment of chromium to the cell when tagging is done prior to freezing and thawing.

An *in vitro* examination of the blood platelets showed them to be substantially reduced in number as anticipated inasmuch as special precautions for platelet handling were not observed. Those remaining in whole blood after freezing and thawing appeared morphologically normal but were present in insufficient number for any test of function.

The following determinations were made on the clotting factors of the supernatant plasma: prothrombin time, recalcification time, accelerator globulin, antihemophilic factor, plasma thromboplastic component and serum prothrombin conversion accelerator. All were unaltered within the limits of experimental error compared to determinations on the same blood prior to freezing and thawing.

Storage. Only the most preliminary storage experiments have been undertaken. X-ray diffraction studies show complete crystallization in the frozen blood on the basis of which it is assumed that, while storage below about 130°C would probably be virtually indefinite, higher and more practical temperatures should still permit good survival for months or years, limited primarily by the rate of denaturation resulting from the dehydration of freezing. Exposures to temperatures as high as -20°C for a few minutes were found to be without ill effect. Preliminary results from longer storage at -60°C are difficult to interpret because of an accompanying acidification due to exposure to CO_2 from the dry ice coolant. Storage loss at -60°C was initially rather rapid, reaching 15% after 20 days (pH 6.5) but slowing subsequently to less than 2% per week. There is every expectation that the relationship between storage decay and temperature will be in accordance with the Arrhenius expression,

showing a logarithmic-decrease in decay rate with reduction in temperature.

Conclusions. Aside from premature speculation on the application of rapid freezing to blood preservation and storage, it can be unequivocally stated that, on the basis of *in vivo* survival, erythrocytes may be taken to liquid nitrogen temperature and back without destruction. The rather unusual fragility of the blood cell and the close relationship between its fragility and its state of health supports the impression that erythrocytes are particularly difficult to freeze. This in turn lends encouragement to the hope that other biological entities which can meet the rather stringent size requirements can be similarly frozen making available not only the benefits of long term storage, but providing also an intriguing new research tool through the study of transient phenomena interrupted and immobilized by the solid state.

Summary. 1. In accordance with previously derived criteria for non-traumatic rapid freezing, very small (.45-.9 mm) droplets of citrated whole blood with added dextrose are sprayed onto liquid nitrogen. The frozen blood is reconstituted in warm saline. 2. Two human transfusions of frozen and thawed whole blood containing erythrocytes tagged with Cr^{51} demonstrate the ability of red cells to survive *in vivo* after freezing in liquid nitrogen at -195.8°C .

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Antibody Production in BALB/c Mice Following Injection of Lyophilized Tumor S621 in Freund's Adjuvant.*† (22107)

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Instances of isoimmunization of mice with their own tumors have recently been reviewed by Foley(1) and Snell(2). The criterion of immunization was ability of mice to cause regression of a viable implant of the tumor. Evidence was presented previously(3) for ability of strain BALB/c mice to produce antibody to homologous tumor S621. The criteria of antibody formation were ability of mice sensitized with lyophilized tumor to undergo anaphylactic shock on a second, intravenous injection; and ability of mice previously given tail inoculation of viable tumor, with tail and tumor subsequently removed, to cause regression of a second, subcutaneous injection of viable tumor.

The work reported herein was done to determine whether or not injection of lyophilized tumor in Freund's adjuvant provided a sufficient antigenic stimulus for detection of antibody by 3 technics: tumor regression; *in vitro* anaphylaxis by the Schultz-Dale reaction; and diminution of the number of exudate cells when cells were incubated with antigen and complement.

Experimental. Antibody production by protection against tumor growth. 1953 Experiments—Immunization with lyophilized S621: BALB/c mice from the colony of Dr. Flavia Richardson were used. Approximately equal numbers of both sexes, over 4 months of age, were injected intraabdominally as follows: (a) 20 mice injected with lyophilized S621† in Freund's adjuvant prepared as before(3) with the exception that

only 50 mg of tumor were contained in the 0.5 ml injected; (b) 33 mice were injected with a preparation of lyophilized S621 identical with (a) except that the tumor in saline suspension was subjected to sonic treatment for 30 minutes in a Raytheon sonic oscillator immediately prior to mixing with adjuvant; and (c) 26 mice were injected with Freund's adjuvant equal in amount to that contained in the S621-adjuvant mixtures. *Injection of viable S621:* Five weeks following injection of lyophilized tumor, mice were inoculated subcutaneously with viable tumor prepared as follows: Fresh, healthy tumor tissue removed aseptically from host, and suspension of single cells prepared by Snell's sieve method(4). After making a cell count, the suspension was diluted with buffered glucose-Ringer's solution(4) to 150000 cells/ml. Each mouse was then injected subcutaneously with 0.1 ml, equivalent to 15000 cells. Twenty-two normal control mice were also injected. This dose was chosen because previous studies indicated that it exceeded, but not greatly, the minimum dose insuring tumor growth. Mice were examined at intervals for appearance of tumors. Experiment was terminated after 3½ months.

*1955 Experiments—*Mice were BALB/c from the colony of Dr. George Snell. Otherwise, they were comparable in every way to those used in 1953. They were injected with S621 under following conditions: (A) 17 mice were injected intra-abdominally with S621 in Freund's as described previously; (B) 16 mice were injected with the same preparation subcutaneously; (C) 11 mice were injected intraabdominally with 50 mg S621 suspended in 0.5 ml buffered saline; (D) 12 mice were injected with same preparation subcutaneously. Control mice included 11 injected with Freund's adjuvant intra-abdominally; and 14 injected subcutaneously.

* This work was supported by grant-in-aid from American Cancer Society upon recommendation of Committee on Growth of the National Research Council.

† Part of this work was done while senior author was at Roscoe B. Jackson Memorial Laboratory (1953).

‡ A BALB/c fibrosarcoma induced by methylcholanthrene in 1948.

TABLE I. Effect of Prior Injection of Lyophilized S621 in Freund's Adjuvant on Growth of Viable Inoculum.

Material inj.	Date	Route of inj.	Progressively growing tumors; death	Slight, if any initial growth comp. regression	Total* by route	Total*† all routes
S621-Freund's	1953	IA	13/20*	7/20	18/37	32/53
	55	IA	5/17	12/17		
	"	SC	14/16	2/16	14/16	
S621-Freund's sonic	53	IA	28/33	5/33	28/33	28/33
S-621	55	IA	11/11		11/11	23/23
		SC	12/12		12/12	
Freund's	53	IA	25/26‡		36/37	50/51
	55	IA	11/11			
	"	SC	14/14		14/14	
None	53	—	22/22		40/40	40/40
	55	—	18/18			

* No. dead of tumors

No. inoculated

† No sex difference was ever apparent.

‡ One mouse showed moderate initial growth, followed by complete regression.

Five weeks after injection of lyophilized tissue, immunity of mice was challenged by subcutaneous injection of 30000 cells, prepared as before. Eighteen normal mice were injected as controls. Mice were examined as before, and experiment terminated after similar interval.

Results of the 2 experiments are recorded in Table I. They show that immunization of BALB/c mice with lyophilized tumor S621 in Freund's adjuvant provides sufficient antigenic stimulus to protect 21 of 53 mice from growth of a viable inoculum of tumor. When the immunizing injection was given intra-abdominally, over 50% of the mice were immune. Injection of lyophilized S621 in saline did not protect mice against a subsequent injection of viable tumor. Sonic treatment of lyophilized S621 reduced antigenicity, but apparently did not destroy it.

Evidence of antibody production by in vitro anaphylaxis. Fifteen female BALB/c Jax mice were immunized with 50 mg of S621 in Freund's adjuvant as before. After 1 or 2 months, and 3 to 4 weeks before testing, they were given a small booster dose of S621 subcutaneously. Included in these experiments were 6 female survivors from the 1955

experiment previously reported. The technic for testing anaphylactic sensitivity in mouse uteri by the Schultz-Dale technic of *in vitro* anaphylaxis has been described(5). The test antigen was prepared from either lyophilized or fresh tumor. Lyophilized tumor was made to concentration of 50 mg/ml in special Ringer's solution(5), ground in glass, and the supernatant fluid, after centrifugation for 10 minutes at 3500 rpm, was used as antigen. Fresh tumor, which in general proved a little more potent antigen in this test, was prepared in like manner, with a crude attempt to approximate the concentration of lyophilized material. All materials were kept at 0°-4°C, and aseptic technic was used while preparing the antigen. After killing the mice and putting uterine strips in 45-ml water bath, 1.0 or 0.5 ml of antigen was added, and the reaction recorded on a kymograph. After contraction of the tissue, a second addition of antigen was made to test desensitization, and reactivity of the tissue tested by addition of 10 µg/ml of acetylcholine. Of the 15 BALB/c Jax mice tested, 8 gave positive reactions on at least two strips of uterus, and 7 gave negative reactions. Five of the 6 BALB/c Snell mice which survived the inoculation of viable cells

following immunization, gave positive reactions. Uterine strips from 3 normal BALB/c mice gave no reaction with either fresh or lyophilized antigen.

Evidence of antibody production by in vitro lysis of cells from sensitized mice. To become thoroughly familiar with the technic involved, preliminary work was done using egg white as antigen, as described elsewhere (6). Twenty-seven BALB/c Jax mice were immunized with 50 mg of lyophilized S621 in Freund's adjuvant, and tested 5 to 6 weeks later. Peritoneal exudate cells were obtained as follows: 2 ml of sterile mineral oil was injected intra-abdominally; 16-18 hours later, 2 ml of sterile heparinized serum-Fox solution (6) was injected. Ten minutes later cells were removed by aspiration, and put into chilled siliconed tubes. The cells were washed twice at 0°-4°C with serum-Fox solution, and resuspended in it to a concentration to give a count of 5000-20000 cells/mm³. The S621 antigen used in the test was the supernatant fluid obtained after 50 mg/ml of lyophilized tumor had been ground in Fox solution and centrifuged 10 minutes at 3500 rpm. The control antigen was normal, lyophilized BALB/c liver, prepared in identical manner. Cells from 27 mice were tested individually. In each test, 6 siliconed tubes were set up, and the materials added to each in 0.1-ml amounts. Tubes 1 and 2 contained cells, S621, and complement;§ the controls contained serum-Fox solution instead of complement. The other 3 tubes were comparable except liver antigen was used instead of S621. After all additions had been made to each tube, which was shaken 100 times, a standard cell count made in National Bureau of Standards certified pipettes was counted in a certified counting chamber. The 6 tubes were then incubated for 2 hours at 37°C, after which time they were again shaken and another count made, using for each tube the same pipette. Data were collected for each of the 27 mice, and recorded as the percent difference in number of cells from 0 to 2

TABLE II. Effect of Antigen and Complement on Peritoneal Exudate Cells from 27 S621 Sensitized BALB/c Jax Mice.

	Mean*	Stand. dev.	Difference†	t
Tests 1 and 2 (S621 antigen)	-11.4	7.71		
S621 control	3.0	7.63	14.4	6.73‡
Tests 3 and 4 (Liver antigen)	.9	7.76	12.3	5.75‡
Liver control	3.1	8.23	14.1	6.55‡

* Avg % difference in No. of cells at 0 time and 2 hr.

† Mean divergence from tests 1 and 2.

‡ Significant beyond .001 level of confidence.

hours. For statistical analysis, results of duplicate tubes for each antigen, which showed good agreement in both cases, were averaged. Mean and standard deviation for each group were determined; and the statistical difference between experimental and control groups was determined by the *t* test. Results of this analysis are recorded in Table II.

From the results expressed in Table II, it can be seen that peritoneal exudate cells from mice sensitized by injection of tumor S621 in Freund's adjuvant were subject to lysis in the presence of antigen and complement; and that no such lysis occurred when normal tissue antigen from liver was used instead of the tumor.

Discussion. Work reported in a previous paper showed that strain BALB/c mice were capable of forming antibody to their homologous tumor S621. After injection of viable tumor in the tail, mice caused regression of a second implant of viable tumor. Some survivors from this experiment, as well as mice immunized by injection of lyophilized tumor in Freund's adjuvant, or lyophilized tumor alone followed by adrenalectomy, were anaphylactically sensitive to an antigen derived from S621.

Experiments here reported lend further support to the concept of formation of these antibodies. Mice immunized with lyophilized tumor tissue in Freund's adjuvant caused regression of subsequent viable implants; and uteri from female survivors of this experiment, as well as from other similarly immu-

§ The 1-10000 dilution of commercial lyophilized guinea pig serum was tested many times, and never had any deleterious effect on the cells.

nized mice, were anaphylactically sensitive as tested by the Schultz-Dale technic. The exudate cells of other mice, also sensitized with S621 in Freund's adjuvant, were lysed *in vitro* in the presence of the S621 antigen and complement.

With the exception of the technic of tumor regression, all our methods for detection of antibodies—*in vivo* anaphylaxis, *in vitro* anaphylaxis, and specific immune lysis of cells—are generally accepted immunological methods to detect "hypersensitive" antibodies, which in either immediate or delayed type of hypersensitivity reactions are believed to be in close association with cells. Recent work of Cole and Favour (7) has shown that in hypersensitivity of the guinea pig to tubercle bacilli, antibodies responsible for delayed "tuberculin" type hypersensitivity, as well as those of anaphylactic type, occur in the serum. Repeated attempts to find antibodies in circulation of BALB/c mice sensitized with S621 by various technics—precipitation, complement-fixation, hemagglutination of cells, which have or have not been treated with tannic acid prior to treatment with antigen, and passive protection with serum from immunized mice—have so far failed. Other studies of anaphylaxis in mice (8,9) indicate that a circulating, bivalent antibody is not the antibody concerned in anaphylaxis in the mouse. This leads to the tentative hypothesis that the antibody concerned in immunity to tumor S621 in BALB/c mice is not the conventional bivalent antibody, and even though it may be in circulation, is an antibody in close association with tissue cells.

Stewart (10) has presented a survey of spontaneous regression of various types of malignancies in man, many of which were accompanied by the classical signs of hypersensitivity in the host at time of regression of the tumor.

Immunity to S621 usually is accompanied by a state of hypersensitivity, but hypersensitivity can apparently exist in absence of sufficient immunity to protect the animal

from viable cell growth. In a previous paper (3), as well as in present experiments, it is noteworthy that mice immunized with the lyophilized tissue in the absence of the adjuvant were not capable of causing regression of a viable implant of tumor, even though they had antibody demonstrable by the more sensitive anaphylactic technic. It is quite possible that lyophilized tissue alone did not provide sufficient antigenic stimulation for the formation of enough antibody to protect against relatively large challenge inocula.

Summary. 1. Antibodies were demonstrated in BALB/c mice which had received an injection of lyophilized homologous tumor S621 in Freund's adjuvant by the following technics: tumor regression; *in vitro* anaphylaxis using the Schultz-Dale reaction; decrease in the number of exudate cells when cells were incubated with antigen and complement. 2. Mice which recovered from a challenge injection of viable tumor were shown to react when tested by the Schultz-Dale technic.

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Inhibition Studies with *Streptococcus faecalis*. Xanthones and Thiaxanthones. (22108)

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Woods' finding(1), that the chemotherapeutic properties of sulfonamides resided in their ability to interfere with the utilization of p-aminobenzoic acid by the organism, has given tremendous impetus to the study of antimetabolites. Such studies, of course, may be approached from more than one direction. For example, anti-folics were prepared on the basis of their studied dissimilarity to the structure of folic acid. This gave rise to the useful agents aminopterin(2) and amethopterin(3). From another approach we have examined compounds prepared(4-6) for use against schistosomiasis(7) for their ability to interfere with utilization of folic acid by *Streptococcus faecalis*. The results of such tests on 16 xanthones and 63 thiaxanthones are discussed below.

Methods. The medium used for these studies with *S. faecalis* (ATCC No. 8043) was slightly modified from that of Teply and Elvehjem(8). The modifications consisted of omitting xanthine and of using "vitamin-free" casein hydrolysate (Nutritional Biochemicals) in place of charcoal-treated material suggested by the authors. Under these conditions the blanks required about 0.5 ml of N/10 alkali per tube for neutralization. Upon

addition of 1 mμg of folic acid per ml of medium, growth of the organism, as judged by acid production after 72 hours, was represented by 9.5 to 10 ml of N/10 alkali per tube. Half maximum growth of the organism, the level against which the test compounds were run, resulted from addition of 0.32 mμg of folic acid per ml of medium. For the tests the compounds were added to duplicate tubes at levels of 1.6, 8.0, 40 or 200 μg per ml of medium. Tubes were plugged, sterilized at 120°C for about 12 minutes, cooled, inoculated with suspension of the organism(9), and incubated at 37°C for 3 days. Growth of the organism was estimated titrimetrically.

Results. The results summarized in Tables I-III are expressed as an arbitrary ratio term. The upper (and smaller) value represents μg of compound per ml of medium which had little or no inhibitory effect on growth of the organism. The lower (and larger) figure represents amount of test compound which completely or essentially completely inhibited growth of the organism. By way of reference, the ratio terms for some known anti-folics under these conditions of test were: (μg/ml) aminopterin 0.008/0.04, amethopterin 0.00032/0.0016, and pyrimeth-

TABLE I. Results of Tests with 16 Xanthones against *S. faecalis* in the Presence of Limiting Amounts of Folic Acid.

Compound,	8 1 7 6 5 CH=CH-CH-CO-CH=CH CH=CH-CH-O-CH-CH=CH 2 3	Derivatives	Activity vs. folic acid, μg/ml
1-NH(CH ₂) ₂ N(C ₂ H ₅) ₂		4-CH ₃ 6-OCH ₃ , 4,6-(CH ₃) ₂	8/ 40
N(C ₂ H ₅) CH ₂ CHOH		4-Cl 6-CH ₃ , 4,6-(CH ₃) ₂ , 4-CH ₃ 6-Cl, 4-CH ₃ 6-OCH ₃ (4)	"
NHCH ₂ CHOHCH ₃		4-CH ₃ (4)	40/200
N(CH ₃) CH ₂ CHOH CH ₃		"	"
NH CH ₂ CHOH C ₂ H ₅		4-CH ₃ 6-Cl(4)	8/ 40
NHCH ₂ C(OH) (CH ₃) ₂		"	0
N(C ₂ H ₅) CH ₂ CHOHCH ₃		4-CH ₃ (4)	40/200
N(C ₂ H ₅) CH ₂ CHOHCH ₃		6-Cl(4)	8/ 40
N(C ₄ H ₉) CH ₂ CHOHCH ₃		(4)	"
N(C ₂ H ₅) CH ₂ C(OH) (CH ₃) ₂		6-Cl(4)	0
		"	20/100
		"	0

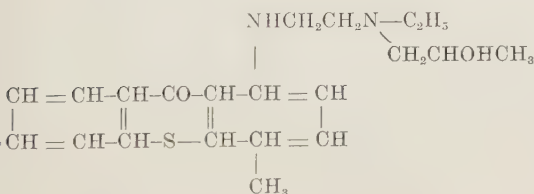
TABLE II. Thiaxanthenes Which Inhibit *S. faecalis* in the Presence of Limiting Amounts of Folic Acid.

Compound,	$\begin{array}{c} 8 \\ 7 \text{ CH}=\text{CH}-\text{CH}-\text{CO}-\text{CH}-\text{CH}=\text{CH} \text{ 2} \\ \quad \quad \quad \quad \quad \quad \\ 6 \text{ CH}=\text{CH}-\text{CH}-\text{S}-\text{CH}-\text{CH}=\text{CH} \text{ 3} \\ 5 \quad \quad \quad 4 \end{array}$	Derivatives	Activity vs. folic acid, $\mu\text{g/ml}$
1-NH(CH ₂) ₂ NHCH ₂ CH ₂ OH		4-CH ₃	8/ 40
NHCH ₂ CHOH CH ₃		(5), 4-CH ₃ 6-Cl	" "
NHCH ₂ CHOH CH ₂ OH		4-CH ₃ (6)	"
NH CH ₂ CHOH C ₂ H ₅		4-CH ₃	"
"			
NH CH ₂ C(OH) (CH ₃) ₂		6-Cl(5)	1.6/ 8
"		(5)	8/ 40
N(CH ₃) CH ₂ CHOHCH ₃		6-Cl	1.6/ 8
NC ₅ H ₁₀		(5)	8/ 40
N(CH ₃) ₂		6-Cl	1.6/ 8
N(C ₂ H ₅) ₂		4-CH ₃	8/ 40
N(C ₂ H ₅)CH ₂ CH ₂ OH		(6), 4,6-(CH ₃) ₂	"
"		(5), 6-Cl(6), 4,6-(CH ₃) ₂ , 4-CH ₃ 6-OCH ₃ , 4-CH ₃ 6-Br	"
N(C ₂ H ₅) CH ₂ CHOH CH ₃		6-Cl(5)	1.6/ 40
N(C ₄ H ₉) CH ₂ CH ₂ OH		"	1.6/ 8
N(C ₄ H ₉) ₂		(5)	8/ 40
NC ₃ H ₁₀		4,7-(CH ₃) ₂ (6)	40/200
1-NH(CH ₂) ₃ N(C ₆ H ₁₃) ₂		4-CH ₃	8/ 40
1-NHCH(CH ₃) CH ₂ N(C ₂ H ₅) ₂		(6)	"
1-NH(CH ₂) ₃ N(C ₂ H ₅) ₂		"	"
1-NH(CH ₂) ₃ N(C ₂ H ₅) CH ₂ CH ₂ OH		4-CH ₃ 7-Cl (6)	1.6/ 8
1-NHCH ₂ CHOH CH ₂ N(C ₂ H ₅) ₂		(6)	8/ 40
"		4-CH ₃ 7-Cl (6)	"
1-NH(CH ₂) ₃ NH C ₃ H ₇		2,4-(CH ₃) ₂ (6)	40/200
N(C ₃ H ₇) ₂		4-CH ₃ (6)	8/ 40
N(C ₃ H ₁₁) ₂		"	"
1-NH(CH ₂) ₄ N(C ₂ H ₅) ₂		"	"

amine 0.0016/0.008.

From the data summarized in the Tables it may be seen that 13 of the 16 xanthenes and 35 of the 63 thiaxanthenes manifested some degree of toxicity to the organism. Since thymidine has been shown to abolish toxicity of some compounds which block utilization of folic acid(10), thymidine at 2 μg per ml of medium, was added in place of folic acid. The nucleoside level was otherwise adequate for optimum growth with the above medium.

In the thymidine-containing medium all xanthenes and thiaxanthenes inhibitory in the presence of folic acid were still inhibitory, with one exception. The exception was



Further tests showed that thymine(11,12) was unable to replace thymidine in abolishing the toxicity of this compound. This thiaxanthone thus differed from aminopterin, amethopterin, and pyrimethamine, which in the medium used, exhibited no adverse effects on growth of *S. faecalis* in the presence of thymine. The latter fact has been noted previously by others(13). On the other hand, the behavior of this thiaxanthone may be considered similar to that noted by Shive *et al.* (10) with another organism, who observed that thymidine, but not thymine, was able to abolish toxicity of either the sulfonamides or 2,4-diamino-6,7-diphenylpteridine for *L. arabinosus* 17-5. Whether or not the unusual metabolic blocking property of 6-chloro-1-{2-[ethyl - (2 - hydroxypropyl) amino]ethylamino}-4-methylthiaxanthone will prove useful alone or in combination with other blocking agents must await further studies under way.

In contradistinction to the unusual proper-

TABLE III. Thioxanthenes Which Do Not Interfere with the Utilization of Folic Acid by *S. faecalis*.

Compound,	8		1		Derivatives
	7	CH=CH-CH-CO-CH-CH=CH ₂	2		
	6	CH=CH-CH-S-CH-CH=CH ₂	3		
	5		4		
1-NH(CH ₂) ₂ N(CH ₂)CH ₂ CHOHCH ₃					4-CH ₃ 6-Cl (5)
NC ₂ H ₅ O					4-CH ₃ 6-Cl (6)
N(C ₂ H ₅) ₂					4-NO ₂ 6-Cl (6)
N(C ₂ H ₅)CH ₂ CH ₂ OH					4-CH ₃ 5-Cl (6)
N(C ₂ H ₅)CH ₂ C(OH)(CH ₃) ₂					6-Cl (5)
N(C ₃ H ₇) ₂					(6)
N(C ₄ H ₉)CH ₂ CHOHCH ₃					4-CH ₃ 6-Cl (5)
N(C ₄ H ₉) ₂					4-NO ₂ , 4,6-Cl ₂ , 4,6-(CH ₃) ₂ , 5-Cl(6), 4-CH ₃ 6-Cl, 4-CH ₃ 7-Cl(6), 7-NO ₂ (6), 4,5,7-(CH ₃) ₃ , 4-Cl 5-CH ₃ (6), 4-Cl 5,7-(CH ₃) ₂ , 4-Cl 7-NO ₂ , 4-NHCOCH ₃ (6), 4-NHCOC ₆ H ₅ (6), 4-NHSO ₂ C ₆ H ₅ (6)
N(C ₂ H ₅) ₂					4-CH ₃ (6)
N(C ₃ H ₇) ₂					"
N(C ₄ H ₉) ₂					"
1-NH(CH ₂) ₃ N(C ₄ H ₉) ₂					"

ties of thioxanthone above, its xanthone analog given in Table I exhibited no adverse effects on the organism in the presence of folic acid.

It may be noted that the 4 thioxanthenes indicated by Berberian, Dennis, and Freele (7) to be schistosomacidal behaved quite differently from each other when examined for toxicity with *S. faecalis*. Thymidine was unable to reverse the toxicity of one (6-chloro-1-{2-[ethyl-(2-hydroxyethyl)amino]ethylamino}-4-methylthioxanthone). Thymidine abolished the toxicity of one (6-chloro-1-{2-[ethyl(2-hydroxypropyl)amino]ethylamino}-4-methylthioxanthone), as indicated above. The remaining two (1-(2-dibutylaminoethylamino)-4-methylthioxanthone) and (6-chloro-1-[2-ethyl-2-(2-hydroxy-2-methylpropylamino)ethyl-amino]-4-methylthioxanthone) exhibited no adverse effects on growth of the organism. Presumably, therefore, the schistosomacidal properties of the foregoing 4 thioxanthenes do not reside in their ability to interfere in the nucleic acid metabolism of *S. mansoni*.

We may conclude, accordingly, that one compound of interest, 6-chloro-1-{2-[ethyl(2-hydroxypropyl)amino]ethylamino}-4-methylthioxanthone, has been brought to the fore by our study. While it does not compare favorably as an anti-folic with known anti-folics,

being only 1/200, or less, as active as some of the better known ones, its differing pattern of inhibition points it up as a compound of possible interest and application, alone or in combination with other blocking agents.

Summary. 1. A series of xanthenes and thioxanthenes initially prepared for tests against schistosomiasis was tested for toxicity with *S. faecalis* in the presence of limiting amounts of folic acid. Under these conditions 13 of the 16 xanthenes and 35 of the 63 thioxanthenes tested were toxic. The toxicity of only one thioxanthone could be abolished. 2. 6-Chloro-1-{2-[ethyl(2-hydroxypropyl)amino]ethylamino}-4-methylthioxanthone was inert with thymidine in the medium. Thymine was not able to replace thymidine in this respect. 3. Separately, it may be indicated that no correlation was discerned between activity against *S. faecalis* and *S. mansoni* infection in mice.

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Neutralizing Antibody to Viruses of Poliomyelitis in Sera of Domestic Animals. (22109)

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The explanation for the origin of seasonal outbreaks of poliomyelitis in the temperate zone is unknown, although it is now evident that man to man transmission is responsible for maintenance of an epidemic. Viral isolation studies have, as yet, failed to implicate animal-reservoir-insect-vector pattern which is so characteristic of many other neurotropic viruses. However, most isolation studies were done prior to the development of tissue culture technics, and intensive surveys were limited. Immunological studies have been carried out by Hammon(1) and others(2,3) in which "neutralizing factors" or "protective substances" have been found in sera of various animals. The status of these "protective substances" is uncertain for they were demonstrable only in low dilution and against one strain (Lansing, type 2), the only strain readily available in most laboratories. Whether one is here dealing with a non-specific neutralizing substance or specific antibody is a critical point, for identification of these substances as specific antibodies to viruses of poliomyelitis would point strongly to the existence of either (1) an extra-human reservoir of viruses of poliomyelitis or (2) a serologically related group of viruses. In the present study animal sera were tested for presence of neutralizing antibody to all 3

types of poliomyelitis virus using the HeLa cell. The protective substance existing in certain cases was identified as a true antibody. To determine the origin of this antibody, extensive though unsuccessful attempts were made to isolate the agents responsible for production of this antibody.

Methods. The HeLa Cell. Techniques employed in the management of the HeLa cell have been adequately described(4), and were followed in the present study. The HeLa cells were routinely grown in rubber stoppered test tubes in a growth medium consisting of 40% human adult serum, 2.5% chick embryo extract and 57.5% Hanks' balanced salt solution. Before use in neutralization test, the cells were washed 3 times with balanced salt solution and 0.9 ml maintenance solution was added to each test tube. The maintenance medium was composed of 90% maintenance solution as described by Syverton, Scherer and Ellwood(4) and 10% chicken serum. *Strains of Poliovirus.* The Brunhilde (type 1), YSK (type 2) and Leon (type 3) strains were employed as representatives of the 3 known antigenic types of poliovirus. These viruses were used in a 100 tissue culture dose concentration against each animal sera tested. *Source and Preparation of Serum Samples.* Table I illustrates the source of sera and geographical origin of animals from which sera were obtained. Sam-

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TABLE I. Number and Source of Animal Sera.

Animal	No. of sera	Source	Geographical origin
Cows	20	Slaughter house	Pa.
Steer	22	<i>Idem</i>	Midwest
Calves	18	"	Va.
Horses	28	Vet. School, Univ. Pa.	Pa. & N.Y.
Hogs	19	Slaughter house	Midwest
Chickens	20	Poultry market	Pa.
Lambs	19	Slaughter house	Va.
Dogs	26	Temple Med. School	Pa.
Cats	5	<i>Idem</i>	Unknown

ples of blood were collected in sterile tubes, allowed to clot, and refrigerated overnight. Serum was drawn off, inactivated at 56°C for one hour, and stored in a dry-ice box for approximately 6 months before testing.

Neutralization Test. Serum dilutions were placed in sterile test tubes in 0.4 ml amounts to which was added 0.4 ml virus, of either types 1, 2 or 3, containing 100 tissue culture doses. The serum-virus mixtures remained at room temperature for one hour. Neutralization of the virus was determined by inoculating 0.1 ml of the mixture into 3 HeLa tubes. Tissue, animal serum toxicity and virus controls were routinely run. After 96 hours at 36°C the tubes were read for cytopathogenic effects. Highest dilution of serum completely inhibiting action of virus was the neutralizing titer of the serum. To determine if the neutralizing action was associated with the globulin fraction of the sera, globulins were precipitated out with a saturated solution of ammonium sulfate. The precipitated globulin was then diluted with distilled water to a final volume equal to that of original volume of sera. This was dialyzed and tested to determine if it had retained its protective activity against viruses of poliomyelitis. **Sources and Preparation of Tissue Specimens for Viral Isolation Studies.** In an attempt to establish the presence of a virus that might explain the origin of antibodies to viruses of poliomyelitis, samples of spinal cord, spleen, lymph node and feces were collected from cows, steers and calves. Specimens were collected as follows: approximately 1 cm² tissue, or 10 ml of feces were collected, 10 specimens from 10 animals pooled, and a 50% suspension prepared by grinding with mortar and pestle

in tryptose broth containing 300 units/ml penicillin and 300 µg/ml dihydrostreptomycin. The suspension was then centrifuged at 3500-4000 rpm for 30 minutes, and the supernatant fluid tested for viral activity. Some tissue specimens proved to be toxic to HeLa cells, therefore the supernatant fluid of centrifuged suspensions was further diluted 1-5 in Hanks' balanced salt solution to reduce toxicity of test materials. One-tenth ml of each specimen was inoculated into 3 HeLa cultures in test tubes containing 0.9 ml maintenance solution. These tubes were incubated at 36°C and observed for cellular changes. After 5 days the fluid medium from each set of 3 cultures was pooled and 0.1 ml of this was inoculated into each of 3 HeLa cultures. This second passage also served further to dilute out toxic factors. After 4 days incubation at 36°C HeLa cultures were again observed for cytopathogenic changes. In addition, tissues were stained and observed for inclusion bodies to detect any agent which might have multiplied without producing a gross cytopathogenic effect. After removal of the media, HeLa cultures were stained directly in test tubes and on coverslips from Porter flask cultures with May-Grunwald-Giemsa stain.

Results. The data presented in Table II indicate type and number of animal sera having neutralizing activity against poliovirus. At a serum dilution of 1:2 some in-

TABLE II. Neutralizing Activity of Animal Sera against Polioviruses Types 1, 2 or 3.

Animal	No. of sera tested	No. of animals with neutralizing activity against polioviruses*	
		Neutralization titer 1:2	1:10 or greater
Bovine			
Cow	20	5	14
Steer	22	3	6
Calf	18	5	0
Lamb	19	5	0
Chicken	20	6	2
Hog	19	3	6
Horse	28	6	9
Dog	30	5	0
Cat	5	0	0

* An animal serum was listed as a single positive if it neutralized types 1, 2 or 3 polioviruses or any combination of the 3 types.

TABLE III. Type Distribution of Neutralizing Activity against Poliomyelitis Viruses, Types 1, 2, and 3.

Animal	No. of sera tested	No. of positive reactions*			Total No. of positive reactions†
		1	2	3	
Bovine					
Cow	20	8	12	4	24
Steer	22	1	5	0	6
Calf	18	0	0	0	0
Horse	28	5	6	0	11
Hog	19	2	3	1	6
Chicken	20	1	1	0	2

* Only those sera exhibiting a neutralization titer of 1:10 or greater were included among the positive reactions.

† A single positive serum specimen may have contributed 1, 2 or 3 positive reactions depending on its ability to neutralize 1, 2 or 3 types of poliomyelitis virus.

dividuals of each species tested showed neutralizing activity, with exception of the cat. In many cases this neutralizing activity was lost upon further dilution. Since the neutralizing activity of undiluted or only slightly diluted serum may have been the expression of a non-specific inhibitory substance, only those species whose sera showed at least occasional activity at dilutions of 1:10 or greater were considered as possible poliomyelitis antibody producers. It will be seen in Table II that cows, steers, horses, hogs, and to a questionable extent, chickens possessed a capacity to neutralize one or more of the 3

TABLE IV. Titers of Antibodies in 20 Cow Sera against Poliomyelitis Viruses, Types 1, 2 and 3.

Cow sera	Titers of sera*		
	1	2	3
1	0	10	0
2	10	10	0
3	50	10	0
4	0	250	0
5	10	50	0
6	10	0	10
7	50	10	50
8	0	0	10
9	10	10	0
10	0	10	0
11	50	10	0
12	0	50	0
13	10	50	10
14	0	10	0
15-20	0	0	0
Total No. positive	8	12	4

* Expressed as reciprocal of actual dilution.

types of poliomyelitis virus in serum dilutions of 1:10 or greater. On the other hand, sera of calves, lambs, dogs and cats were consistently negative at dilutions of 1:10 or higher, hence were not considered to be related to specific antibody. Type distribution of positive sera in the cow and steer is indicated in Table III. It will be seen that neutralizing activity against type 2 poliovirus was most frequently encountered followed by type 1, and least frequently against type 3.

Probably the strongest evidence indicating that these neutralizing substances are antibodies is the pattern of neutralization observed with cow and steer sera as indicated in Tables IV and V. It can be seen that titers vary from 1:10 to 1:250 (the highest dilution tested) and that the pattern of neutraliza-

TABLE V. Titers of Antibodies in Steer Sera against Poliomyelitis Viruses, Types 1, 2 and 3.

Steer sera	Titers of sera*		
	1	2	3
1	0	250	0
2	0	50	0
3	0	250	0
4	0	250	0
5	0	250	0
6	10	0	0
7-22	0	0	0
Total positive	1	5	0

* Expressed as reciprocal of actual dilution.

tion tends to differ with each animal. Some animals have antibody to type 1, some to type 2, some to type 3, and still others have various combinations of these antibodies. This pattern, by definition, rules out the possibility of neutralizing action being due to a non-specific inhibitory substance. As further evidence that these are antibodies resulting from a specific antigenic stimulus and not a natural antibody it should be noted (Table III) that with increasing age there is an increasing ability to neutralize viruses of poliomyelitis. Thus, 70% of the cows (7-14 yrs), 27% of steers (1-2 yrs) and none of the calves (3-4½ months) had neutralizing antibodies in dilutions of 1:10 or higher. This increase with age is the typical pattern observed in antibody development. Table VI illustrates that this neutralizing activity is

TABLE VI. Neutralizing Activity of Pooled, Positive Animal Sera and the Resuspended Globulin Fraction against Types 1, 2 and 3 Polioviruses.

Animal sera, pooled	Neutralizing activity					
	Before globulin precipitation			Resuspended globulin fraction		
	1	Type 2	3	1	Type 2	3
Cow	+	+	+	+	+	+
Steer	+	+	—	+	+	—
Horse	+	+	—	+	+	—
Hog	+	+	+	—	+	—
Chicken	+	+	—	—	—	—

(+) Neutralization. (—) No neutralization.

retained in the precipitated globulin fraction of sera.

Though extensive studies were done with bovine sera, preliminary data has been included on neutralizing activity of horse, hog and chicken sera (Table VII). It will be noted that both horse and hog sera were often able to neutralize in high dilutions, while chicken sera were able to neutralize only in low dilutions. These limited studies do suggest that sera of horses and hogs also possess specific neutralizing antibodies to viruses of poliomyelitis.

Viral Isolation Studies. One possible interpretation of the antibodies observed (see discussion) is the assumption that cows may be infected with all three types of poliovirus. If this assumption is valid, it should be possible to isolate the virus of poliomyelitis from the bovine species. Preliminary isolation studies have been carried out and no viruses were isolated in HeLa cells which were neu-

TABLE VII. Neutralizing Activity Exhibited by Horse, Hog, and Chicken Sera against Types 1, 2 and 3 Polioviruses.

Animal	No. of animals	No. of animals positive	Antipoliovirus titer and type distribution of positive sera*								
			1:10			Titer 1:50			1:250		
			1	2	3	Type 1	2	3	1	2	3
Horse	28	9	3	3	0	2	3	0	0	0	0
Hog	19	6	1	2	1	1	0	0	0	1	0
Chicken	20	2	1	1	0	0	0	0	0	0	0

* A single positive serum specimen may have contributed 1, 2 or 3 positive reactions depending on its ability to neutralize 1, 2 or 3 types of poliomyelitis virus.

tralized by types 1, 2 or 3 poliomyelitis antibody. These studies were carried out with materials collected from cows, steers and calves; 470 spinal cords, 330 spleens, 310 lymph nodes and 410 feces specimens were studied as indicated under Methods.

Discussion. Our data indicate that neutralizing substances in bovine species are true antibodies for the reasons summarized below. 1. Antibody patterns in cows are highly specific, e.g., some cows have no antibody, some have antibody to types 1 and 2, some to types 1 and 3, others to types 2 and 3, and still others have antibody to types 1, 2 and 3. This high degree of specificity, by definition rules out any non-specific neutralizing substance. 2. These are not natural antibodies for in addition to reasons stated above they are absent in young animals, increasing with age, and are not present in all adults of the species. In addition, titers are frequently high (1:250 dilution of serum neutralizes 100 TCD of virus). 3. Neutralizing substances are not inactivated by heating at 56°C for 30 minutes and are found in the globulin fraction of the serum.

It is therefore concluded that the neutralizing substances in cow sera are antibodies specific for polioviruses. It is important to note that these are antibodies *against* viruses of poliomyelitis and are not necessarily a result of infection with human strains of poliovirus.

While this study was in progress it was found that Dr. Albert Sabin (personal communication) was also carrying out a somewhat similar study(5). Dr. Sabin's data also indicate that neutralizing substances present in bovine sera are specific antibodies.

What is the origin of the antigenic stimulus? There are two possibilities: (1) These are antibodies resulting from subclinical infection of cows with human strains of poliovirus, with the implication that viruses of poliomyelitis exist and multiply in areas outside of man. (2). These antibodies arose not from the antigenic stimulus of human poliomyelitis strains but rather from viruses of non-human origin that are serologically related to human viruses. It is obvious that

such strains, if isolated, may be important as immunizing agents against poliovirus in humans. There is little experimental data to help us choose between these alternatives and little will be gained by further discussion of the possibilities since proof in either case requires the isolation from animals of the specific infectious agent.

Summary. (1) The sera of cows and steers were shown to possess neutralizing substances in high titer against the viruses of poliomyelitis. These have been shown to be specific antibodies against the viruses of poliomyelitis. (2) The sera of horses, hogs and chickens also possessed modest neutralizing activity against the viruses of poliomyelitis. (3) The sera of dogs, cats, calves and lambs do not

possess neutralizing activity against the viruses of poliomyelitis. (4) Attempts to isolate a virus from the bovine species that might explain the origin of these antibodies were unsuccessful. The antigenic stimulus giving rise to antibodies against the viruses of poliomyelitis remains unknown.

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Myasthenia gravis. I. Importance of Potassium in Inhibitory Action of Normal and Myasthenic Thymus Extracts.* (22110)

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To investigate the concept that myasthenia gravis is due to a circulating blocking agent possibly elaborated by the thymus (McEachern)(1) several investigators(2-5) have tested thymus extracts on nerve-muscle preparations from various animals.

The present study was undertaken to repeat Dr. Wilson's experiments with the eventual objective of isolating the active blocking substance. The isolated frog sartorius nerve-muscle preparation, which had been used successfully by Wilson and Stoner(6) to detect the blocking activity of serum from myasthenic patients, was used in this investigation rather than the rat phrenic nerve-diaphragm preparation.

Methods. A. Isolated Frog Sartorius Preparation: Frogs were pithed and the sartorius muscle excised with a small block of underlying muscle to support the motor nerve. Frog Ringer, containing 6.5 g NaCl,

0.14 g KCl, 0.12 g CaCl₂, 0.2 g NaHCO₃ and 0.01 g NaH₂PO₄/l, bathed the muscle during dissection. After excision, the muscle was placed at normal resting length (5 to 10 g tension) in a plastic chamber of 2 ml capacity, provided with two sets of silver electrodes to permit independent direct and indirect stimulation of the muscle and nerve respectively. The small size of this chamber is of considerable advantage when only small volumes of test solution are available. The mechanical myogram was obtained by a transducer, consisting of an aluminum bar (6.5 x 1.2 x 0.015 cm) on either side of which was cemented a strain gage element.[†] The myogram was recorded with a Sanborn strain gage recorder (Model 127) fed from a Sanborn strain gage amplifier (Model 140). This arrangement permitted recording of the isometric myogram. All experiments were performed at 23 to 25°C. Thresholds for nerve and muscle excitability were recorded at beginning of each experiment. After 15 minutes equilibra-

* This study was supported by a grant from the Institute of Neurological Diseases and Blindness, National Institutes of Health.

[†] Baldwin-Lima-Hamilton Corp., Type A7.

tion of the muscle in oxygenated frog Ringer, the Ringer solution was removed and the muscle stimulated via the motor nerve for 5 seconds with 0.5 to 1 volt monophasic stimuli of 0.5 millisecond duration at a frequency of 2 cps. A second and third control series of contractions were obtained at 5 minute intervals before adding test solution to the muscle chamber. At one to 3 minute intervals, the test solution was removed from the chamber and the muscle stimulated as before. When the muscle failed to respond to indirect stimulation, it was stimulated directly with 5 second trains of stimuli of increasing voltage (1,3,5,10,14 volts). The muscle was then repeatedly washed with frog Ringer and recovery of muscle tension was followed for 60 minutes. Average amplitude of 10 consecutive tracings was computed and plotted against time. Activity of each extract was calculated on the basis of time for 50% inhibition of contraction strength per g of extracted thymus tissue.

B. *Method of extraction* was slightly modified after the method of Wilson, Obrist and Wilson(5). Thymus glands, removed at operation from patients with myasthenia gravis, were weighed and placed in cold acetone (4°C) after representative blocks had been taken for preparation of sections for microscopic examination. Each gland was chopped into small pieces and ground in a mortar with 25 ml volumes of cold acetone (4°C) until total extract volume of 1000 ml was obtained. Acetone extract (AE) was filtered and the residual insoluble material (AI) collected and dried to constant weight *in vacuo* over CaCl_2 . The acetone fraction was evaporated to dryness *in vacuo* in a water bath at 37°C. After evaporation, the AE fraction which consisted of a yellow oil, was taken up in 5 ml of Frog Ringer. The red-brown, AI fraction was similarly taken up in 5 ml of frog Ringer. After adjusting the pH of the 2 extracts to the pH of frog Ringer, they were separately tested on the isolated frog sartorius preparation. A fresh sartorius muscle was used in each test. Sixteen thymuses from myasthenic patients, one normal child's thymus, 3 spleens and 2 samples of skeletal muscle

were extracted and tested by this procedure. C. *Heat Inactivation and Dialysis Experiments*: Solutions of acetone insoluble fraction were placed in cellophane bags and dialyzed against frog Ringer. Aliquots of internal and external solutions were subsequently tested. Extracts of both acetone soluble and insoluble fractions were boiled for 2 minutes, cooled and tested on the sartorius preparation.

D. *Experiments with d-Tubocurarine, CaCl_2 and KCl*: d-Tubocurarine (0.003 to 15 γ /ml, and KCl (4 to 40 meq/L.) solutions were tested on the sartorius preparation. The effect of thymus extracts containing added CaCl_2 was also determined. E. The potassium concentration of 16 extracts was quantitatively determined by flame photometry.† Extracts of the acetone soluble fraction were ashed before determination of their potassium content because of fat in the solutions. F. *Thymus Pathology*: The presence of hyperplasia and germinal center formation was recorded (Castleman and Norris)(7) and the relative amounts of parenchyma, fatty and fibrous tissue was independently rated by two observers who were unaware of the activity of the extracts prepared from the glands.

Observations: Control studies performed

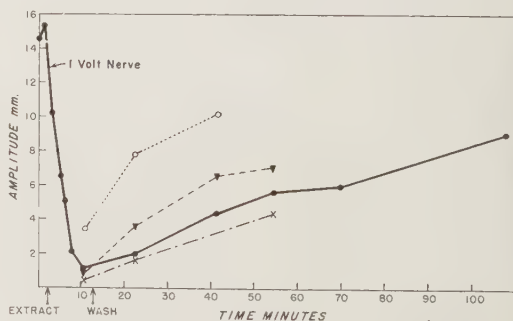


FIG. 1. Heavy circles illustrate decrease in contraction force of indirectly stimulated (1 volt) muscle resulting from immersion in thymus extract. After repeated washing, the contraction strength gradually returns. Crosses (1 volt), triangles (5 volts) and open circles (10 volts) illustrate that the muscle shows impaired response to direct stimulation. After washing, the curve of recovery of the directly stimulated muscle parallels the recovery curve of the indirectly stimulated muscle.

† Acknowledgment is due to Dr. Charles H. DuToit for these determinations.

TABLE I. Clinical Status of 16 Myasthenic Patients and the Activity of Extracts of Their Thymus Glands.

Age	Severity	Duration of symptoms	Activity of acetone insoluble fraction, min./g /50% inhibition
21	Mild	2.5 yr	1.33
25	"	1.5	.51
13	Moderate	3.5	.91
23	"	3.0	2.60
21	"	2.0	.20
15	"	1.5	1.33
16	"	1.0	.67
17	"	3 mo	1.14
19	"	5 yr	.73
30	Severe	7	.66
23	"	1	1.53
41	"	6	2.74 T*
13	"	2	.29
46	"	Months ?	.38 T
24	"	4.5 yr	.67
18	"	2	2.05

* T = Tumor.

with several changes of frog Ringer over periods of 60 to 70 minutes showed a maximal decrease in the tension curve of 15 to 20% of initial tension. When tissue extracts were tested, the average experiment rarely exceeded 30 minutes. Thus, decreases of the tension curve of greater magnitude are attributed to the inhibitory effects of the extracts. A dose-response curve constructed for several concentrations of d-Tubocurarine (0.003 to 15 γ /ml) showed that concentrations of 0.03 γ /ml of d-Tubocurarine could be accurately detected by this method. As expected, the muscle responded to direct stimulation; it failed to respond to indirect stimulation.

Experiments with Tissue Extracts: Both acetone soluble and acetone insoluble fractions of all thymus glands tested depressed the tension curve of indirectly stimulated sartorii in 2 to 20 minutes. However, when muscles failed to respond to indirect stimulation they also failed to respond to direct stimulation (Fig. 1). After 30 to 60 minutes of washing with several changes of frog Ringer, the test muscles slowly recovered. The time course of recovery for indirect and direct stimulation was similar (Fig. 1). Thus, it appeared that the extracts acted directly on the muscle, although action at the neuromuscular junction could not be excluded.

Great variability of activity of extracts occurred which failed to correlate with age of patient, duration of symptoms or clinical severity of the disease. (Table I). A normal child's thymus possessed considerable inhibitory activity as did extracts of 3 spleens and 2 samples of human skeletal muscle. Extracts of these tissues rendered the test muscle inexcitable by indirect stimulation in 5 to 10 minutes. At this time, the muscles uniformly failed to respond to direct stimulation. The time course of recovery of the muscle after washing with several changes of frog Ringer was similar to that observed with thymus extracts.

Dialysis of Extracts: After dialysis of the acetone insoluble fraction, most of the inhibitory activity was present in the solution outside the cellophane membrane. This material produced a decrease of tension curves of both indirectly and directly stimulated muscles.

Heated Extracts: The acetone insoluble extract retained its inhibitory activity after boiling for 2 minutes.

Potassium concentrations of thymus extracts ranged from 2 to 33 times the concentration of potassium in frog Ringer. When the extract containing highest concentration of potassium (66 mEq/L.) was added to the test muscle, spontaneous fasciculations of the muscle occurred. These concentrations of potassium are 33 times the normal potassium content of frog Ringer. Table II shows a rough correlation between time for 50% inhibition of the muscle by the acetone-insoluble fraction and a poor correlation with the acetone soluble fraction.

Effect of Added Potassium Chloride. When

TABLE II. Potassium Content and Activity of Thymus Extracts.

Per g P ₅₀	(K ⁺) P	TP ₅₀	TA ₅₀	(K ⁺) A
.727	2.0	8.0	—	—
.254	4.8	6.5	5.2	7.2
1.140	8.0	11.2	12.0	—
.671	11.0	8.0	3.0	7.7
.381	13.0	5.0	6.0	7.11
2.05	13.6	8.5	10.5	20.0
.287	17.0	8.8	1.9	6.1
2.740	38.5	3.7	4.7	24.3
1.330	42.0	2.9	—	—
.675	66.0	2.6	8.7	34.0

TABLE III. Potassium Content of Ten Thymuses Compared with Histological Architecture.

K content acetone soluble fraction, mEq/L	K content acetone insoluble fraction, mEq/L	Parenchyma	Fibrosis	Fat	Germinal center formation
2.0	—	1+	3+	2+	A*
4.8	—	3+	<1+	2+	P
8.0	—	3+	2+	1+	P
11.0	7.7	2+	3+	1+	P
13.0	7.7	3+	2+	2+	T
13.6	20.0	2+	2+	1+	A
17.0	6.1	4+	1+	1+	P
38.5	24.3	4+	1+	2+	T
42.0	—	4+	1+	1+	P
66.0	8.7	3+	0+	1+	A

* A = Absent; P = Present; T = Tumor.

frog Ringer solutions containing added potassium chloride were applied to frog sartorius preparations, the effects produced were similar to those obtained with thymus, spleen and striated muscle extracts. Low concentrations of added potassium (1.28 to 12.8 mEq/L.) gradually reduced tension response of test muscle to indirect and direct stimulation. The activity of these solutions roughly paralleled activity of thymus extracts containing comparable concentrations of potassium. A T_{50} of 2.9 minutes was obtained when a thymus extract containing 40 mEq/L. of potassium was tested whereas a T_{50} of 4.5 minutes was obtained when a frog Ringer solution containing 40 mEq/L of added potassium was tested. The time course of recovery of the tension curve after washing with frog Ringer was similar to that observed after tissue extracts had been tested on the sartorius preparation. Addition of CaCl_2 to thymus extracts (5 to 10 times normal concentration of CaCl_2 in frog Ringer) decreased the inhibitory effects of the extracts on the tension curve of frog sartorii. In a typical experiment, the T_{50} of the acetone insoluble fraction was 2.6 minutes whereas after addition of CaCl_2 (total concentration— 5×10^{-3} M) the T_{50} increased to 8.5 minutes.

Thymus Gland Pathology. In 15 glands tested, 9 showed hyperplasia and germinal center formation, 4 failed to show these changes and 2 were thymomas. Table III shows that thymus glands with the most parenchyma possessed the greatest inhibitory activity.

Discussion. The original intention of re-

peating previously published experiments concerning the muscle inhibiting effects of thymus extracts (Wilson, Obrist, and Wilson) (5) as a point of departure for isolation of the active material led to investigation of potassium content of acetone-extracts of thymus tissue. The finding of a heat stable, dialysable substance in thymus extracts and extracts of other tissues which rendered the test nerve-muscle preparation inexcitable on indirect and direct stimulation suggested that potassium might be responsible for the observed inhibitory action. Presence of potassium concentrations ranging from 2 to 44 times normal potassium content of frog Ringer confirms the idea that potassium alone could be responsible for the inhibition observed in our experiments and in experiments reported by others (Wilson, Obrist and Wilson) (5). Furthermore, correlation between potassium concentration of thymus and other extracts and their ability to depress tension curve of the frog sartorius preparation strongly suggests that this is the case. Duplication of these effects by adding equivalent concentrations of KCl to frog Ringer solutions constitutes additional evidence that potassium concentrations which occurred in thymus extracts can produce rapid decrease of muscle tension. Failure of activity of acetone-soluble fraction to correlate well with potassium content is probably due to the protective effect of lipids in the test solution.

The deleterious effect of increased potassium concentrations on muscle excitability has long been recognized (8-10). According to Gellhorn's data (10) at the sodium concen-

tration used in our experiments (0.134 M), concentrations of potassium chloride in excess of $96 \times 10^{-4}\text{ M}$ produce rapid loss of excitability. Table II shows that the thymus extracts tested contained 48 to $660 \times 10^{-4}\text{ M}$ of potassium. Gellhorn(10) showed that calcium ions also antagonized the inhibitory effect of increased potassium concentrations. Approximately equal amounts of CaCl_2 above the concentration ($8.1 \times 10^{-4}\text{ M}$) of CaCl_2 in frog Ringer antagonized approximately equal amounts of KCl added to normal frog Ringer solution. Thus, in experiments where CaCl_2 was added to thymus extracts, the decrease in inhibitory activity of the extract can be attributed to antagonism between high concentrations of potassium in the extracts and the added calcium ions.

It should be emphasized that the concentrated tissue extracts used contain other ions (Na^+ , Ca^{++} , Mg^{++}) in undetermined concentrations which, because of abnormal ion ratios, may also contribute to inhibitory action of these extracts on isolated frog nerve muscle preparation. Therefore, we cannot conclude that elevated potassium concentrations alone are responsible for the inhibition observed. Furthermore, we cannot conclude that extracts of myasthenic thymus glands prepared in this way are unique in containing considerable quantities of potassium. There is no evidence to indicate that myasthenic thymus glands contain more tissue potassium than normal thymus glands of comparable cellularity. Since the extracts tested in this study were prepared according to the methods of Wilson and associates(5), it is probable that the results reported by these investigators may be partly, if not entirely, due to high concentration of potassium in the extracts. The combined acetone soluble and acetone insoluble extracts used by Wilson and associates may have contained considerable concentrations of potassium.

It is of interest that in our study a rough correlation was found between cellularity of thymus glands tested, their potassium content and the activity of the extracts. Extracts prepared from glands which contained little fatty and fibrous tissue contained more

extractable potassium and possessed greater activity. Thus, it might be expected that a myasthenic or a normal child's thymus might contain more inhibitory activity than the fatty, fibrous glands of adults as reported by Wilson and associates(5). However, it is not possible to explain on this basis the positive correlation between extract activity and the post-operative course of the patients reported by these investigators. The contractures observed by Wilson and associates(5) when high concentrations of thymus extracts were added to frog rectus abdominis muscle preparations may have been due to potassium in the extracts(11-13). The potentiation of acetylcholine action on the frog rectus preparation by thymus extracts reported by Wilson and associates(5) may also be due to potassium ions in the extracts.

Since simple acetone and saline extraction of tissue breis results in crude mixtures of many components including small ions in varying proportions, extreme caution must be exercised in interpreting the effects produced by these mixtures on nerve-muscle preparations. Of particular importance is potassium which in low concentrations blocks the neuromuscular junction (Overton)(14) and in high concentrations abolishes the excitability of the muscle.

It is possible that the inhibitory activity of myasthenic thymus extracts may not entirely be due to potassium in the extracts, but inhibitory activity not due to potassium cannot be determined in the presence of excessive potassium concentrations or abnormal concentrations of other ions in the test solutions. Although desalting procedures might eliminate this source of error, it is probable that other extraction methods should be used in searching for inhibitory activity in thymus glands from patients with myasthenia gravis.

Summary. 1. A method is described which permits recording of the isolated myogram of indirectly or directly stimulated isolated frog sartorius muscle. 2. Acetone extracts prepared from thymus glands obtained from 16 myasthenic patients, one normal child's thymus, 3 spleens and 2 samples of striated muscle were tested on the isolated frog sartorius

preparation. 3. Both acetone soluble and acetone insoluble fractions markedly decrease the muscle tension curve in 2 to 20 minutes. When the muscle becomes inexcitable on indirect stimulation, it is also inexcitable on direct stimulation. 4. The active material is heat stable, dialysable and is antagonized by CaCl_2 added to the extracts. 5. Concentrations of potassium 2 to 44 times the potassium concentration of frog Ringer were found in the extracts. These concentrations of potassium are sufficient to produce the muscle inhibition observed. 6. The significance of these results relevant to the search for a blocking agent in the thymus glands of myasthenic patients is discussed.

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Serum Complement in Hepatobiliary Diseases.* (22111)

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There is considerable suggestive evidence in the literature that the liver may be involved in the formation of serum complement and that the latter is diminished in liver disease and in experimental hepatic injury. Reports prior to 1929 were reviewed by Goldner(1). They indicate diminution of serum complement (C') in experimental phosphorus or alcohol poisoning and its disappearance following hepatectomy in rabbits. In perfusion experiments with heat-inactivated serum, guinea pig liver contributed complement to perfusing serum, enabling the latter to hemolyze sheep red cells. On the other hand, complement remained normal in abdominally eviscerated dogs which had retained their liv-

ers. Goldner, using a rabbit hemolysin-sheep cell system in which complete hemolysis was used for the C' titer, found that C' was subnormal in 8 of 20 cases of "icterus catarrhalis" and in 10 of 11 cases of (choolangitic and portal) cirrhosis; furthermore, 3 of 7 cases of metastatic tumor of the liver showed diminution or absence of complement, one case of acute yellow atrophy complete disappearance of C' , while 6 cases of cholecystitis revealed no change from normal. Recently, Jordan(2), using his hematocrit method, reported marked diminution of C' in all of 18 cases of cirrhosis studied; 22 cases of hepatitis showed some drop in C' near the peak of the disease, while 12 patients with obstructive jaundice yielded high values. In contrast to these findings, Dulaney(3), using a 50%-hemolysis endpoint as determined pho-

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TABLE I. Serum Complement in 65 Patients with Hepatobiliary Disease.

Diagnosis	No. of patients	Age of patients, yr		Serum bilirubin, mg/100 ml		Serum complement, units	
		Range	Mean	Range	Mean	Range	Mean
Acute hepatitis							
Virus A or B	16 (3)	13-60	30	1.1-31.2	14.7	1.4-5.7	3.2
Leptospiral	3	37-52	44	12.0-22.8	18.3	3.8-4.1	3.9
Toxic	1		22		38.8		1.2
Cirrhosis							
Alcoholic	18 (12)	25-56	40	.4-27.0	5.3	1.2-4.7	2.8
Posthepatic	2	47, 75		3.5, 9.6		.8, .8	
Biliary obstruction							
Benign	9*	28-60	48	.8-28.0	9.9	2.0-6.1	4.0
Malignant	16†	39-72	57	.8-34.0	9.6	1.9-4.9	3.5

() denotes presence of complicating conditions.

* Acute (2 cases), chronic cholecystitis (4), common duct stone (3).

† Carcinoma of gastrointestinal tract (4 cases), pancreas (9), biliary tract (2) and kidney (1), causing jaundice and/or metastatic hepatomegaly.

tometrically, detected no significant changes in C' titer in 25 patients with liver disease, including 5 cases of infectious hepatitis and 12 of cirrhosis. However, in the course of the same investigation, this author found C' diminished in each of 24 patients with induced malaria.

In an attempt to clarify this apparent conflict in the literature, the present study was undertaken.

Materials and methods. Collection of all blood specimens was done in Atlanta† from subjects in pre-prandial state. Only those patients were included whose diagnoses appeared established either by autopsy, biopsy, surgical operation, or prolonged clinical observation. Twenty healthy persons served as normal controls, 65 patients had various forms of liver or biliary tract diseases (Table I), and 25 patients had miscellaneous illnesses. These included pneumonia (3 cases), acute rheumatic fever (5), infectious mononucleosis (1), aseptic meningitis (1), chronic pancreatitis (3), rheumatoid arthritis (1), sickle cell anemia (2), sarcoidosis (1), lymphoma (1), multiple myeloma (1), myocardial infarct (1), malignant hypertension (1), disseminated lupus (1) and acute (1) and burned out stage of glomerulonephritis (2). Promptly upon separation from the clots, the sera were stored at -40°C and, packed with

dry ice, were transported by air to New York for analysis (K.L.), by the method previously reported(4). Fifty % hemolysis was used as endpoint. A serum which produced 50% hemolysis in the same dilution as a standard 1:8 guinea pig serum was said to contain 1 unit of C'. Obviously then, a serum which produced 50% hemolysis at twice the dilution of the standard serum contained 2 units, etc. In our experience the simultaneous testing of a standard guinea pig serum in each test represents an indispensable control over many variables, especially the changing fragility of the sheep cells.

Results. The serum complement ranged in the normal control group from 1.5 to 4.4 units, with a mean of 2.7. Corresponding figures for the abnormal control group, exclusive of one case of acute glomerulonephritis and one of disseminated lupus, were 1.4-4.7, and 2.9. The C' titer of the latter 2 cases was severely diminished to 0.1 and 0.5 unit, respectively. In previous studies involving 166 control cases, one of the authors (K.L.) had found a normal range of 1.1 to 3 units with an average of 1.7(5). Hence, for classification of the present results, 1.1 units was taken as the lower limit of normal and 1.1 to 1.4 units were considered as borderline range. Two distinctly subnormal values in the hepatobiliary disease group (Table I) were obtained in the 2 patients with post-hepatic cirrhosis, 1 and 2 weeks, respec-

† Grady Memorial Hospital, Emory University Hospital and Vet. Adm. Hospital.

tively, before their deaths from hepatic insufficiency. Three other cases of uncomplicated portal cirrhosis with ascites and low serum proteins had borderline titers. C' titers in all other cases tabulated were either within or above the normal range. While these values represent initial examinations upon admission to the hospital, the majority of these patients had one or more follow-up determinations; in no instance was a subnormal value encountered.

Discussion. It is a well accepted fact that inflammation may result in a rise of C' (4,6,10-13). This fact seems to be borne out by the cases of benign obstructive jaundice, many of whom had an accompanying cholecystitis and who showed the highest mean C' values, as well as by the majority of the hepatitis patients, who showed elevated C' values. From the data presented, it appears that hepatocellular disease does not lower the C' titer. The slight diminution of C' in the 2 cases of posthepatic cirrhosis should probably be rather attributed to the moribund state of these patients than to the presence of hepatic disease. A drop in C' associated with extremely grave conditions, including overwhelming infections has been observed by other authors(1,3,7).

The discrepancy between our data and those of Goldner(1) and of Jordan(2) cannot be readily resolved, but may be justifiably attributed to differences in analytical techniques. Certainly, the method used in this study could be trusted to reveal evidence of subnormal C' levels, which it has uncovered with great consistency in acute and subacute glomerulonephritis, in nephrosis and in disseminated lupus erythematosus(4,5).

The observed absence of a C' drop in hepatitis would suggest that there is no widespread antigen-antibody reaction taking place in this disease, in contrast to glomerular diseases and to hypersensitivity states, in which such a reaction has been postulated(4,5,6-9).

The lack of a C' diminution in hepatocellular disease speaks against formation of C' —

or, at least, its limiting component—in or through the liver† and tends to shift attention to the reticulo-endothelial system as its physiologic source. In contradistinction to Jordan(2), who speaks of “the great discriminatory power of the complement test,” our data do not indicate that this offers any aid in the differential diagnosis of liver disease, with the exception, perhaps, of a grave prognosis in the occasional event of C' diminution.

Summary. The serum complement titer was determined in 20 patients with acute hepatitis, 20 with hepatic cirrhosis, and 25 with (benign and malignant) biliary obstruction. The only distinctly subnormal values were encountered in 2 moribund patients suffering from cirrhosis. The data speak against formation of complement in or through the liver and fail to indicate usefulness of complement titration in diagnosis of hepatobiliary diseases.

† Authors found C' elevated in 2 patients in whom about two-thirds of the liver had been surgically removed because of hepatoma.

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Interaction of 16 Epi-Estriol and Estradiol 17 β on Uterine Growth.*† (22112)

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In a previous publication the authors reported on estrogenic activity of 16 epi-estriol, an estrogen recently extracted from urine of pregnant women by Marrian and Bauld(1). Our results showed conclusively that this estrogen was slightly less active than estriol (2). Since estriol was shown to have the ability to interfere with uterine growth-promoting action of estradiol 17 β (3), it seemed of considerable interest to determine the effects of 16 epi-estriol on estradiol 17 β -induced uterine growth.

Therefore, the present study takes for its central theme a series of experiments designed to elucidate the effects of 16 epi-estriol on uterine growth-promoting and vaginal cornifying ability of estradiol 17 β .

Procedures. Virgin, albino rats, approximately 100 days of age, were used. They were started on treatment 7 days after bilateral ovariectomy. The 16 epi-estriol was dissolved in U.S.P. propylene glycol, and estradiol 17 β was dissolved in sesame oil and/or propylene glycol. They were injected subcutaneously, each of these compounds at a separate site, daily for 3 days. Vaginal smears were taken daily, and 72 hours after first injection necropsies were performed. Uteri were removed quickly and slit longitudinally on bibulous paper, thus removing excess uterine luminal fluid. The uteri were then weighed to nearest 0.2 mg on a Roller-Smith torsion balance. After wet weights were determined, the uteri were placed in an oven at 110°C for dry weight

determinations. A standard period of 24 hours at 110°C was used uniformly for drying each uterus. After this period the weight of each uterus was determined on a Volland Analytical Balance.

Results. These results clearly indicate that 5 μ g of 16 epi-estriol are required to demonstrably restrict in part the uterine growth promoting action of 0.1 μ g estradiol 17 β on both a wet and a dry weight basis. Smaller doses of 16 epiestriol when given in combination with our standard dose of 0.1 μ g estradiol 17 β do not seem to appreciably modify the response of the uterus to the more potent estrogen (Table I). On the other hand it seems quite apparent that a marked reduction in uterine growth is effected when 10 μ g or more of 16 epi-estriol are administered concurrently, but at different injection sites, with the estradiol 17 β (Table I). Actually, a maximal re-

TABLE I. Influence of 16 Epi-estriol on Uterine Growth Promoting Effects of Estradiol 17 β in Ovariectomized Rats.

Treatment	No. of animals	Uterine wet wt, mg	Uterine dry wt, mg
Ovariectomized controls, no treatment	25	115.5 $\pm$ 3.3†	22.4 $\pm$.7
.1 μ g E*	42	227.3 $\pm$ 6.1	42.3 $\pm$ 1.8
1.0 μ g 16 epi + .1 μ g E	21	214.2 $\pm$ 6.5	39.5 $\pm$ 4.7
2.0 μ g 16 epi + .1 μ g E	7	203.7 $\pm$ 11.1	37.6 $\pm$ 3.2
5.0 μ g 16 epi + .1 μ g E	7	190.5 $\pm$ 9.5	35.1 $\pm$ 2.6
8.0 μ g 16 epi + .1 μ g E	13	191.4 $\pm$ 7.3	35.0 $\pm$ 2.2
10.0 μ g 16 epi + .1 μ g E	6	174.9 $\pm$ 6.5	34.9 $\pm$ 1.0
25.0 μ g 16 epi + .1 μ g E	7	173.3 $\pm$ 8.8	33.7 $\pm$ 2.1
Reference controls: 16 epi alone			
1.0 μ g	8	150.0 $\pm$ 8.6	26.1 $\pm$.8
5.0 "	8	142.7 $\pm$ 6.8	25.8 $\pm$ 1.7
10.0 "	9	171.4 $\pm$ 8.7	28.5 $\pm$ 1.6
25.0 "	9	164.3 $\pm$ 5.1	30.5 $\pm$ 1.1

* E = Estradiol 17 β ; epi = epi-estriol.

† Avg $\pm$ stand. error.

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striction of uterine growth is obtained on the combined treatment of 0.1 μ g estradiol 17 β plus 10 μ g 16 epi-estriol. Of particular significance is the fact that a summation of the two estrogens does not obtain, even though the maximal growth potential of the uterus is at least 50% more than that reported for 0.1 μ g estradiol 17 β (3).

This substance does not seem to alter the vaginal cornifying effects of estradiol 17 β . The vaginal smears of those animals on 0.1 μ g estradiol 17 β with and without 16 epi-estriol seem to be relatively indistinguishable, both showing a preponderance of cornified epithelial cells with degenerative nuclei. Thus, the restrictive action of 16 epi-estriol seems to be mainly on the uterine growth-promoting effects of estradiol 17 β .

These results quite clearly parallel those of Hisaw, Velardo, and Goolsby(3), who reported that estriol likewise inhibited estradiol 17 β on the growth of the uterus. Furthermore, Huggins and Jensen(4) showed that 16 epi-estriol was quite efficacious in restricting the effects of estrone on the uterus in hypophysectomized rats. Therefore, these results seem wholly consonant with the thought that the various estrogens and/or their metabolites act *in concert* to produce the end organ result (3). These experiments provide additional data that give credence to the concept that an endocrine response is accomplished through

a precise ratio of the hormone and/or its metabolites.

Summary. The adult, ovariectomized rat was used to ascertain the effects of a new estrogen, 16 epi-estriol, on the activity of estradiol 17 β . These results indicate that when 3 daily doses of 5 to 25 μ g of 16 epi-estriol are subcutaneously injected into rats receiving 0.1 μ g estradiol 17 β daily for the 3-day period, the response of the uterus to estradiol is restricted. Normally, 3 daily doses of 0.1 μ g estradiol 17 β increase the uterine wet weight from the value of the ovariectomized rat from approximately 116 mg to 227 mg. The simultaneous administration of 5, 10, or 25 μ g of 16 epi-estriol in addition to the estradiol treatment reduces the response to 191 down to 173 mg. The inhibitory action is likewise reflected in the dry weight data. No apparent modification of the vaginal cornifying action of estradiol could be induced by 16 epi-estriol.

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Hemolysis of Sheep Erythrocytes Sensitized with Leptospiral Extracts. (22113)

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Serodiagnosis of leptospirosis presently is based on agglutination, agglutination-lysis (AL), and complement-fixation procedures. The agglutination test employing killed leptospirae has been found to parallel the AL test in its type specificity(1). Gochenour(1) found that complement-fixation tests employing sonically-disrupted leptospiral antigens(2) showed a broader spectrum than AL procedures. Various modifications of AL

procedure, such as that of Stoenner(3), may alleviate some difficulties associated with AL technic, in this case the hazard of working with living leptospirae. However, several serotypes of leptospirae known to be involved in leptospirosis make it necessary to employ several antigens in the above technics used as screening tests, the choice of serotypes depending on specificity of procedure and the purpose of the investigation(4). The need

for screening procedure employing antigen with specificity sufficiently broad to manifest antibodies produced against all serotypes of leptospirae would seem apparent. Recent progress was made by Chang and McComb (5), who described a hemagglutination (HA) procedure using human blood group O erythrocytes. Their HA test exhibited fairly broad specificity, based upon titers which were, however, very moderate in comparison with those of the highly type-specific AL reaction. Several reports(6-10) appeared concerning microbial substances capable of sensitizing erythrocytes in the presence of homologous antiserum to lytic action of complement (C'). Preliminary studies in this laboratory demonstrated that ethanol extracts of leptospirae were capable of sensitizing sheep erythrocytes to subsequent agglutination by homologous rabbit antisera, confirming the work of Chang (5) using human group O cells. Further investigation showed that sensitized sheep erythrocytes were susceptible to lysis in the presence of high dilutions of homologous rabbit antisera and complement.

This report describes the performance of the hemolytic test (HL) and results of studies to ascertain its specificity and sensitivity in comparison with the AL and HA procedures.

Materials and methods. Cultures—*L. pomona*, *L. icterohemorrhagiae B*, *L. bataviae*, *L. grippo-typhosa*, *L. hebdomadis*, and *L. canicola B* were obtained from Dr. Harold S. Bryan, College of Veterinary Medicine, University of Illinois. *L. autumnalis*, *L. icterohemorrhagiae C*, *L. canicola C* were obtained from Dr. R. S. Chang, Harvard University School of Public Health. *L. canicola R*, *L. hyos*, *L. ballum*, and *L. pyrogenes* were obtained from the Veterinary Division, Army Medical Service Graduate School. All cultures were grown in a medium consisting of 0.2% sterile tryptose-phosphate broth (Difco). Before use, sterile rabbit serum was added to final concentration of 10% and final medium heated in 56°C waterbath for 30 minutes. This medium was simple to prepare and yielded leptospiral growth comparable with other contemporary leptospiral media. *Diluent*. Phosphate-buffered isotonic saline

was used in AL and HA procedures. It consisted of 0.137 M NaCl, 0.01 M Na₂HPO₄, and 0.003 M KH₂PO₄, yielding pH 7.2. Veronal-buffered saline was prepared as described by Mayer(11) and used as diluent in the HL procedure. *The AL Procedure* followed that of Gochenour(1), except that sera were decimally diluted. *Immune sera* were obtained from rabbits injected with viable 5 to 7-day-old cultures of leptospirae. Before use in HA and HL tests, each serum was inactivated and adsorbed with 0.1 volume of washed sheep erythrocytes. Pre-inoculation sera were collected from each rabbit, and checked for presence of leptospiral antibodies by all 3 procedures. *Leptospiral extracts* were prepared essentially as reported by Chang(5), with the exception that the 90% ethanol precipitate was washed in absolute ethanol, dried, and weighed. Dried, weighed extract was then redissolved in merthiolated saline to final stock concentration of 0.2 mg/ml. In later experiments, incorporation of veronal diluent at pH 7.4 in stock solution maintained extracts at constant activity in the HL test for several months. One fraction has been so maintained with constant activity for over 8 months. Active extracts were prepared from all leptospiral cultures excepting *L. grippo-typhosa* and *L. ballum*, from which extracts have been prepared with at most only slight HL activity. Considerable difference occurred between HL activities of extracts obtained from different culture growths of the same organism. *Sensitization of Erythrocytes for HA and HL Procedures*. One volume of 10% suspension of washed sheep erythrocytes was mixed well with 10 volumes of appropriate dilution of leptospiral extract. Erythrocyte suspension and fraction dilution were prepared in appropriate diluents. The mixture was agitated intermittently for 1 hour in 37°C waterbath, centrifuged, and the packed erythrocytes washed once with diluent. The sensitized erythrocytes were then reconstituted to a 1% suspension in appropriate diluent. *HA Procedure*. One-tenth ml volumes of sensitized erythrocytes were added to 0.4 ml volumes of decimal dilutions of sera. The cell control contained sensitized

TABLE I. Reciprocal Agglutination-Lysis Titrations.*

	Anti-leptospiral rabbit sera											
Leptospiral antigens	<i>autumnalis</i>	<i>ballum</i>	<i>bataviae</i>	<i>canicola B</i>	<i>canicola R</i>	<i>grippotyph.</i>	<i>hebdomadis</i>	<i>hyos</i>	<i>ictero B</i>	<i>ictero C</i>	<i>pomona</i>	<i>pyrogenes</i>
<i>autumnalis</i>	4	1	—	—	—	1	2	—	—	4	2	—
<i>ballum</i>	—	4	—	2	2	—	—	—	1	—	—	4
<i>bataviae</i>	—	—	5	—	—	—	2	1	1	—	—	—
<i>canicola B</i>	—	2	—	4	5	1	1	—	2	—	—	2
<i>canicola C</i>	1	2	—	4	5	2	1	—	3	1	—	2
<i>canicola R</i>	—	2	—	4	5	—	—	—	3	—	—	3
<i>grippotyph.</i>	—	—	—	—	—	4	1	—	1	2	1	—
<i>hebdomadis</i>	1	—	—	—	—	—	5	—	—	—	—	—
<i>hyos</i>	—	—	2	1	—	1	1	5	—	—	—	—
<i>ictero B</i>	1	2	—	3	3	—	—	—	4	—	—	3
<i>ictero C</i>	3	—	—	—	1	—	1	—	1	5	2	—
<i>pomona</i>	2	—	—	—	—	—	—	—	—	2	4	—
<i>pyrogenes</i>	—	4	—	2	2	1	—	1	1	—	—	5

* Titers expressed as logs of highest decimal dilutions showing definite reaction. "—" indicates no reaction in a dilution of 10^{-1} or higher.

erythrocytes and diluent. Serum control contained lowest dilution of serum and non-sensitized erythrocytes as a check on adsorption. Tubes were agitated and incubated at 30°C for 16-20 hours, and read by gently flicking the tube. *HL Procedure.* One-tenth ml volumes of sensitized erythrocytes were added to 0.4 ml volumes of decimal dilutions of sera. Two exact units of guinea pig C' in 0.2 ml volume were added to each tube, followed by addition of 0.3 ml of diluent. Serum control contained non-sensitized in place of sensitized erythrocytes. C' control contained diluent in place of serum, and sensitized cell control contained sensitized erythrocytes plus 0.9 ml of diluent. Tubes were agitated and incubated 1 hour in 37°C waterbath and read for hemolysis in usual manner, recording hemolysis as complete, partial, and negative. *Standardization of Leptospiral Extracts for HA and HL Procedures.* Double dilutions of the extract starting from 0.2 mg/ml were used to sensitize erythrocytes in the HA procedure. Such sensitized erythrocytes were then set up against master dilutions of known homologous immune rabbit sera in HA and HL procedures. Titration of all dilutions of extract against each serum dilution in HL tests was made in presence of 0.2 ml of 1:30 dilution of guinea pig C' per tube, which was consistently found

to represent an excess of C'. The end-point or concentration of extract used in further tests was selected as highest dilution, or smallest amount, above which there was no decrease in activity. Acceptable levels of extract activity demonstrated homologous immune serum HA titers between 1:10 and 1:100 and HL titers of 1:100 to 1:10000. Satisfactory HA and HL activity was usually achieved with extract concentrations ranging from 2.0 to 200 mg/ml. Some extracts in high concentrations hemolyzed erythrocytes in diluent; however, the end-point was always several dilutions higher than that which caused hemolysis. *Titration of C' for Use in HL Procedure.* Using leptospiral extract which had been diluted according to previous standardization and the usual decimal dilutions of homologous immune serum, series of titrations were set up, each with increasing 0.05 ml increments of guinea pig C' diluted 1:30. Volumes of C' ranged from 0.05 to 0.3 ml. The end-point or 1 exact unit of C' was taken as the smallest amount showing complete lysis in highest dilution of immune rabbit serum, and 2 exact units in 0.2 ml volume were used thereafter in HL procedure.

Results. Reciprocal AL titrations between various leptospiral serotypes and their homologous and heterologous antisera were carried out. It is apparent from Table I

TABLE II. Reciprocal Hemolytic Titrations.*

	Anti-leptospiral rabbit sera											
Leptospiral antigens	<i>autumnalis</i>	<i>ballum</i>	<i>bataviae</i>	<i>canicola B</i>	<i>canicola R</i>	<i>grippotyph.</i>	<i>hebdomadis</i>	<i>hyos</i>	<i>ictero B</i>	<i>ictero C</i>	<i>pomona</i>	<i>pyrogenes</i>
<i>autumnalis</i>	3	3	4	3	3	2	4	4	3	2	3	3
<i>bataviae</i>	2	3	3	2	2	2	2	3	2	2	2	2
<i>canicola B</i>	2	3	2	2	2	2	2	3	2	2	2	2
<i>canicola C</i>	3	4	4	3	4	3	4	4	3	3	3	4
<i>canicola R</i>	3	3	3	3	3	2	3	4	3	3	3	3
<i>hebdomadis</i>	2	2	2	2	2	2	2	2	2	2	2	2
<i>hyos</i>	3	3	3	2	3	2	3	3	3	2	2	2
<i>ictero B</i>	3	4	3	3	3	2	3	4	3	3	3	3
<i>ictero C</i>	3	3	4	4	4	3	4	4	4	4	3	4
<i>pomona</i>	3	4	5	4	4	3	4	5	4	5	3	4
<i>pyrogenes</i>	2	2	2	2	2	2	2	2	2	1	2	2

* Titers expressed as logs of highest decimal dilutions showing definite reaction.

that AL reaction is quite type-specific as is generally recognized. The same master dilutions of immune rabbit sera were used to set up similar reciprocal HL titrations (Table II). HL reaction is extremely group specific. Antibodies produced against any one of the leptospiral types reacted broadly with all leptospiral fractions studied. The finding of higher heterologous than homologous titers with some antisera is felt to reflect differences in activity between fractions obtained from different leptospiral types. For example, highly active fractions were obtained consistently from our cultures of *L. pomona*, *L. icterohemorrhagiae*, and *L. canicola C*. However, as mentioned previously, only the slightest activity was obtained from extracts prepared from *L. ballum* and *L. grippotyphosa*. Fractions obtained from *L. pyrogenes* were consistently low in HL activity. On the

basis of results in Tables II and III, *L. pomona* was selected as the culture from which we obtained extracts for use in screening human sera for leptospiral antibodies by HL and HA procedures.

Selected leptospiral extracts were used to sensitize sheep erythrocytes which were then cross-titrated in HA procedure with homologous and heterologous immune rabbit sera (Table III). Although only selected extracts were used in HA titrations, it should be mentioned that, as in HL titration, extracts prepared from *L. ballum*, *L. grippotyphosa*, and *L. pyrogenes* consistently possessed minimal activity or were completely inactive. The HA reaction is apparently similar in spectrum to the hemolytic reaction; however, HA titers were consistently lower, which causes the degree of crossing to appear less significant than in HL procedure.

TABLE III. Reciprocal Hemagglutination Titrations.*

Leptospiral antigens	Anti-leptospiral rabbit sera											
	<i>autumnalis</i>	<i>ballum</i>	<i>bataviae</i>	<i>canicola B</i>	<i>canicola R</i>	<i>grippotyph.</i>	<i>hebdomadis</i>	<i>hyos</i>	<i>ictero B</i>	<i>ictero C</i>	<i>pomona</i>	<i>pyrogenes</i>
<i>autumnalis</i>	2	2	1	2	2	1	2	2	2	1	2	2
<i>canicola C</i>	2	2	2	2	2	1	2	2	2	1	2	2
<i>canicola R</i>	1	1	1	1	1	1	1	1	1	—	1	1
<i>ictero C</i>	2	2	2	2	2	1	2	2	2	1	2	2
<i>pomona</i>	2	2	2	2	2	1	2	2	2	1	2	2

* Titers expressed as logs of highest decimal dilutions showing definite reaction. "—" indicates no reaction in a dilution of 10^{-1} or higher.

TABLE IV. Comparative Titrations on Human Sera.

No. of sera showing result	Result using following titrations:		
	AL	HL	HA
505	—*	—	—
9	+†	—	—
10	—	+	—
23	—	—	+
1	+	+	—
24	—	+	+
0	+	—	+
8	+	+	+
Total sera	580		
Total positive	75	18	43
% positive	12.9%	3.1%	7.4%
			9.5%

* Negative = No reaction in dilution of 10^{-1} or higher.

† Positive = Complete reaction in dilution of 10^{-1} or higher.

To compare relative activity of each of the 3 procedures, AL, HA, and HL titrations were performed on 580 human sera submitted at random from hospitals and clinics in this area (Table IV). With none of the patients was an initial clinical diagnosis of leptospirosis considered. All 3 procedures were performed as previously described, and the AL test was performed using 3 types: *L. pomona*, *L. ictero B*, and *L. canicola B*. It should be mentioned that of the 580 sera, only 2 febrile patients were available for repeat serum titrations. One patient showed progressively increasing titers to both AL and HL procedures to peak at 1:10000 levels. The AL titers were against *L. icterohemorrhagiae* and *L. canicola*, and the HA titer remained at 1:10 throughout. The other patient never showed a positive AL titer with any of the 3 leptospirae used as antigens; however, the HL titer progressively increased during course of the illness to a level of 1:10000, and the HA titer rose to a level of 1:100. Six of the positive sera showed AL titers of 1:1000 or higher, and 3 showed AL titers of 1:10000 or higher. In a similar fashion 21 sera showed HL titers of 1:1000 or higher and 5 were as high as 1:10000. None of the sera showed HA titers above 1:100.

It should be noted that the apparent advantage of the HA procedure in detecting antibodies missed by the other two procedures (23 sera) was based upon an HA reaction in

serum dilutions of only 1:10, without which the positive HA reactions would drop to 1.1%. The reason for this number of weak HA reactions and their connection with leptospirosis is not known. The sera had been adequately adsorbed with sheep erythrocytes, as evidenced by negative controls. In contrast, positive reactions in AL and HL procedures were based predominantly upon complete reactions in serum dilutions of 1:100 to 1:10000.

Ability of HL procedure to detect antibodies in titers of 1:100 or higher missed by AL procedure could be readily explained on the basis of differences in specificity of the reactions. Thus, it is felt that a total of 31 of the 34 sera in Table IV may be showing high HL titers stimulated by serotypes of leptospira other than the 3 used in these AL tests. Ability of the AL test to detect antibodies missed by the other 2 procedures (9 sera) is at present unexplained; however, it is possible that this reflects differences in persistence of antibody levels detectable by the 3 procedures. Clarification of this point would depend upon the difficult task of coupling similar studies on selected human cases of leptospirosis with adequate cultural studies, extending over many months from very early in the course of the illness.

If leptospiral extracts should prove to remain stable over long periods of time with respect to HL activity by other workers, it would seem that the HL procedure would show promise as a convenient and useful tool with which to screen human sera for leptospiral antibodies. Although it requires an extra step in the addition of complement over the HA procedure, it is as easily read, more rapid in performance, and considerably more sensitive. It is certainly less hazardous to the operator than the AL procedure. HL procedure would seem to warrant further study as a screening tool by those laboratories where adequate bacteriological control studies as well as patient followups would be available.

Summary. Leptospiral extracts are capable of sensitizing sheep erythrocytes to the HL action of homologous and heterologous lepto-

spiral antiserum and complement, and description of the HL procedure is presented. The HL procedure was shown to be broadly specific, in sharp contrast to the high degree of type specificity seen in the AL procedure. In comparison with HL titrations, the HA reaction was also found to have broad specificity but to be considerably less sensitive than the HL reaction. All 3 procedures were compared in a study involving 580 randomly selected human sera, from which data it would seem that the HL procedure warrants further study as a possible screening tool for the rapid detection and titration of leptospiral antibodies in human sera.

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Failure of Synthetic 5-Hydroxytryptamine Creatinine Sulfate to Enhance Clot Retraction of Platelet-Poor Human Plasma.* (22114)

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Many platelet constituents with specific effects on the hemostatic mechanism have been recently identified(1). Whether they represent intimate constituents of platelets or are carried by them still requires investigation. These agents include a thromboplastic factor (2); constituents accelerating conversion of prothrombin to thrombin and of fibrinogen to fibrin(3), an antiheparin factor(4), possibly antifibrinolysin(5,6). An additional substance, 5-hydroxytryptamine (serotonin), has been identified in dried platelets preparation (7,8) and shown to liberate from platelets during clotting of human blood(9). Serotonin has been shown to possess vasoconstrictor

and capillary protecting activity(10). Recently, it has also been suggested that serotonin is able to induce clot retraction of platelet-poor bovine and human plasma(11,12). This observation is of considerable importance since clot retraction has been generally considered to be due to intact platelets rather than to a single platelet constituent. This study is an attempt to evaluate the clot retraction promoting effect of the commercially available synthetic derivative of serotonin, 5-hydroxy-tryptamine creatinine sulfate, the use of which is currently recommended in the treatment of the bleeding tendency of thrombocytopenic states. At the same time, observations have been made on the ability of this compound to influence the rate and extent of prothrombin conversion during clotting of platelet-poor plasma.

Materials. Native platelet-poor plasma.

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TABLE I. Effect of 5-Hydroxy-tryptamine Creatinine Sulfate on Clot Retraction and Prothrombin Utilization during Clotting of Undiluted Platelet-Poor Human Plasma.*

Final conc. of 5-hydroxy-tryptamine creatinine sulfate, mg %	Clot retraction, % (24 hr after clotting)	Prothrombin activity of serum, % (1 hr after clotting)
80	10.5	87
40	10	84
20	9.5	86
10	10	92
5	8	81
2.5	8.5	84
1.25	9	93
.6	9.5	93
.3	10	87
.1	10	90
Control†	8	90

* Avg results of 4 experiments.

† Saline solution, 1/10 volume.

Blood was collected from fasting healthy donors with the "double syringe technic" in Silicone-coated syringes through Arquad 2-C coated needles. Blood was transferred to pre-cooled Silicone-coated test tubes and centrifuged in the cold (4°C) at 3,000 for 30 minutes. The upper $\frac{2}{3}$ of supernatant plasma only were then promptly transferred to another pre-cooled Silicone-coated test tube and promptly added to the various dilutions of the substance under study. Silicone-coated pipettes were used in all steps. *Citrated platelet-poor plasma.* Blood was collected with the same technic and transferred to Silicone-coated test tubes containing 1/10 volume of 0.2 M solution of sodium citrate. Blood and anticoagulant were promptly mixed by inverting the tube which had been stoppered with paraffin-coated cork. *Synthetic 5-hydroxy-tryptamine creatinine sulfate*, was made available through the courtesy of Dr. Merrill E. Speeter.† Serial dilutions were made in saline solution, and strengths used are indicated in Tables I and II. *Determination of clot retraction.* Immediately after completion of clotting, clot was rimmed by the technic of pushing a thin glass rod to the bottom and circling it around the clot. Tubes were then incubated in water bath at 37°C for 1 or 3 hours. After visual inspection, vol-

ume of serum expressed when the tube was inverted at an angle of 60° was taken as measure of retraction. *Serum prothrombin time (prothrombin consumption)* was determined by our method(13), 1 or 3 hours after completion of clotting.

Results. 1. Effect of 5-hydroxy-tryptamine creatinine sulfate on clot retraction and prothrombin utilization during clotting of platelet-poor native human plasma. Experiments were made in duplicate. Various concentrations of 5-hydroxy-tryptamine creatinine sulfate in a constant volume of 0.1 ml were transferred to chemically clean glass test tubes kept in water bath at 37°C; 0.9 ml of native platelet-poor human plasma were then added to each tube and the contents mixed. A similar experiment was performed using Silicone-coated test tubes and comparable results were obtained. There was no appreciable and constant effect on the speed of clotting, clot retraction and serum prothrombin activity one and 3 hours respectively after completion of coagulation. There was no appreciable difference in clot retraction 24 hours after clotting between tubes containing 5-hydroxy-tryptamine creatinine sulfate and the control containing 1/10 volume of saline solution, as shown in Table I.

2. Effect of 5-hydroxy-tryptamine creatinine sulfate on clot retraction of diluted native or citrated human plasma. Native or citrated human plasma were diluted 1:5 with sterile 0.85% NaCl solution. 4.5 ml of diluted plasma were added to a series of glass test tubes kept in water bath at 37°C con-

TABLE II. Effect of 5-Hydroxy-tryptamine Creatinine Sulfate on Clot Retraction and Prothrombin Utilization during Clotting of Diluted Platelet-Poor Human Plasma.*

Final conc. of 5-hydroxy-tryptamine creatinine sulfate, mg %	Clot retraction, % (24 hr after clotting)	
	Citrated plasma†	Native plasma
40	74	
20	72	No clot
10	70	
Control‡	72	

* Avg of several experiments.

† Clotted with bovine thrombin.

‡ Containing saline solution, 1/10 volume.

§ Director of Biochemical Research, Upjohn Co.

taining various concentrations of 5-hydroxy-tryptamine creatinine sulfate in a constant volume of 0.5 ml. Diluted native plasma was allowed to clot spontaneously. Clotting often did not occur in some instances when plasma had been made practically platelet-free by centrifugation. Diluted citrated plasma was clotted with the addition of 0.2 ml saline solution containing 60 units of purified bovine thrombin. Clot retraction was observed and volume of separated serum measured 3 and 24 hours after completion of coagulation. Results 24 hours after clotting are presented in Table II. Residual prothrombin activity was negligible because of the high dilution of plasma. There was no demonstrable effect on retraction of the clot.

Discussion. Correll *et al.*(10) studied the thromboplastic activity of serotonin derivatives by investigating their ability to accelerate the clotting time of plasma. Their negative results are confirmed by ours. On the other hand, our results on effect of serotonin derivatives on the retraction of the clot are not confirmatory of those already presented in the literature(11,12). Since, however, it is not known whether synthetic products and 5-hydroxy-tryptamine carried or liberated by platelets are identical in structure and properties, our negative experiment does not prove that platelet serotonin may not contribute to the mechanism of clot retraction, as postulated by Fenichel and Seegers.

Perhaps, explanations might be offered for the discrepancy between our findings and those of other authors. There may be differences in purity of the reagents used. Moreover, the complexity and multiplicity of the factors influencing clot retraction has been emphasized by many authors and recently summarized by Budtz-Olsen(14). Thus, it

is likely that, without extremely careful control of all experimental conditions, results may be at great variance and not constantly reproducible. Since little is known of the ideal conditions under which clot retracts *in vitro* and *in vivo*, the evaluation of the clot promoting activity of a drug remains a hazardous problem.

Summary. Synthetic 5-hydroxy-tryptamine creatinine sulfate failed to enhance clot retraction and prothrombin utilization during clotting of platelet-free human plasma. These results are at variance with recently reported findings.

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Delayed Blood Coagulation in Rabbits with Localized Shwartzman Reaction. (22115)

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In previous work(1) the authors elaborated a procedure indicating the presence or absence of fibrinogen in serum derived from minimally heparinized blood. Subsequently, a correlation between the utilization of fibrinogen and the rheumatic state was attempted. The results seemed to indicate an accelerated fibrinogen polymerization in active rheumatic disease. In order to obtain experimental verification of the reported clinical findings this procedure, described as "fibrinogen polymerization test"(2), was applied to rabbits in which a Shwartzman phenomenon had been produced. Minimally heparinized rabbit blood withdrawn 24 hours after the provocative intravenous injection of endotoxin failed, however, to coagulate within an observation period of 7 or 8 hours so that the test could not be carried out properly, since gross coagulation of the heparinized specimen is a prerequisite. This observation seemed to indicate delayed coagulation independent of the *in vitro* addition of minimal amounts of heparin. Consequently, it was postulated that the Shwartzman phenomenon may be associated with a prolonged Lee-White clotting time. Verification of this assumption was sought in the experiments reported herein.

Materials and methods. Twenty rabbits of either sex, each weighing approximately 2 kg, received 0.5 cc of meningococcus endotoxin* intradermally, followed by the intravenous administration of 2.0 cc of endotoxin 24 hours later. On the 3rd, 4th, 5th and 8th day of the experiment, Lee-White clotting times were determined with blood withdrawn by cardiac puncture. About 15 cc were swiftly aspirated after careful shaving of the precordium and palpating of the point of maximum car-

diac impulse. Only a portion of the aspirated 15 cc were utilized for the Lee-White clotting time, thus leaving a considerable amount of blood within the syringe assumed to contain most of the tissue juice with its thromboplastic properties. Three glass tubes measuring 14 mm x 10 cm were utilized for the determination of the Lee-White clotting time. About 2 cc of blood were placed into each tube. A fourth, graduated centrifuge tube containing 0.2 cc of 0.1 molar solution of sodium citrate was filled up to the 2 cc mark and served for the determination of prothrombin time according to the photo-electric modification of the one-stage Quick method(3). Lee-White clotting times were obtained at room temperature which varied from 22°-26°. The end point at which the clotting time was read coincided with the formation of a solid clot which permitted the complete inversion of the test tube without displacement of the coagulum.

Results. Typical localized Shwartzman reactions were elicited in 16 of the 20 rabbits employed in this study. Characteristic hemorrhagic necrotic lesions at the site of the sensitizing intradermal injection of the endotoxin appeared well before 24 hours had elapsed after the provocative intravenous administration of the meningococcal endotoxin. As seen from Table I, the mean Lee-White clotting time in all 20 animals was 18 minutes on the third day of the experiment, *i.e.* 24 hours

TABLE I. Mean Lee-White Clotting Times and Prothrombin Times of Rabbit Blood Obtained through Cardiac Puncture at Various Stages of Shwartzman Reaction.

	Days after initiation of experiment			
	3rd	4th	5th	8th
Lee-White clotting time in min.	18	8.3	7.4	4.3
Prothrombin time in sec	6.7	4.5	5.7	7.4

*We wish to express our appreciation to Dr. Gregory Shwartzman for providing meningococcus endotoxin for the experiments reported herein.

after the provocative intravenous injection, at which time the Schwartzman reaction was quite pronounced and declined to 8.3, 7.4 and 4.3 minutes on the 4th, 5th and 8th day. No group distinction could be established between the clotting times of the majority of rabbits in which the Schwartzman reaction was visible and the few animals in whom no necrotizing lesion appeared. In a separate group of 10 normal rabbits the clotting time of intracardiac blood was determined at room temperature and found to have a mean value of 5.5 minutes.

Prothrombin times in all animals subjected to the Schwartzman phenomenon appeared to be normal throughout (mean value 6.1 seconds) and did not disclose any definite lengthening, even at the time when the Lee-White clotting times were markedly prolonged.

Discussion. Although no satisfactory explanation of the pathogenesis of the Schwartzman phenomenon has yet been found(4), it is known to be characterized by a hemorrhagic necrosis in the prepared skin site. It seems that the second-provocative-injection of bacterial filtrate leads to injury of the walls of small blood vessels. Moreover, thrombosis of these vessels has been found to be a constant pathological feature(4-6).

The results of our study disclose that the localized Schwartzman reaction is accompanied by a prolongation of the Lee-White clotting time with a mean value of 18 minutes as compared to a normal mean of 5.5 minutes. Although the nature of this prolonged clotting time remains undetermined at present, the release of heparin or heparinoid substances as tissue reaction to anaphylactoid stimuli(7)

seems to offer an explanation. The prolonged clotting time may contribute to the hemorrhagic nature of the Schwartzman reaction. On the other hand, it is not of such magnitude as to prevent the phenomenon as has been achieved with large doses of heparin(8,9), namely 30 mg per kg producing incoagulability of rabbit blood for about 7 hours(6). Nevertheless, it seems to be of interest that rabbit blood which physiologically displays a high degree of hypercoagulability should lose this property when the localized Schwartzman phenomenon is induced. Hypercoagulability of rabbit blood is manifested not only in a relatively short Lee-White clotting time, but also in a rather short prothrombin time (Table I).

Summary. 1. A localized Schwartzman reaction was produced in 20 rabbits. 2. The Lee-White clotting time determined with blood obtained through cardiac puncture was found considerably prolonged when the reaction appeared and gradually returned to normal during the ensuing 6 days.

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Hepatic Damage in Rats Fed Human Foodstuffs.* (22116)

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It is well established that laboratory animals fed one or another deficient diet may develop hepatic lesions resembling Laennec's cirrhosis. These observations have supported the view that an inadequate dietary intake contributes to the development of cirrhosis in human subjects. By and large, however, the experimental diets have had little resemblance to the diets eaten by such patients. The hypothesis that dietary deficiency is important in the pathogenesis of human cirrhosis would be strengthened if similar lesions appeared in animals fed foodstuffs resembling those eaten by patients with this disease. Gillman, Gillman, Mandelstam and Gilbert(1) were able to produce severe hepatic damage in rats fed corn meal and sour milk, a diet similar to that eaten by South African natives who commonly develop disease of the liver. The changes observed in rats included diffuse fatty liver, cirrhosis, and atrophy or hypertrophy of individual hepatic lobes without parenchymal necrosis. It seemed of interest to determine whether experimental animals would develop hepatic changes if fed diets similar to those of patients with cirrhosis of the liver in this country. The experiments to be described suggest that rats fed such diets may develop lesions which bear a similarity to those of human cirrhosis.

Method. Young male albino rats of the Wistar strain obtained from Carworth Farms, New City, N. Y., were housed in individual cages with open meshed bottoms. All rats were fed Purina dog chow and tap water *ad libitum* until the onset of the experimental period, at which time their mean weight was about 110 g. The rats were then fed one of

4 diets *ad libitum* as follows: Diet A (12 rats)—Purina dog chow and tap water, Diet B (45 rats)—spaghetti, olive oil and wine; the wine was diluted with an equal volume of water, Diet C (12 rats)—spaghetti, olive oil and 10% glucose in water, Diet D (12 rats)—spaghetti, olive oil and diluted wine with vitamin supplement. The composition of these diets is given in detail below. In 10 rats fed diet B and in all those fed diets C and D the amount ingested was measured grossly, taking into account spillage. For mechanical reasons the measurement of spillage was only approximate. As a result only a crude estimate of the actual dietary intake was possible. The animals did not have access to their feces or urine. Purina dog chow is composed of meat meal, dried skimmed milk, wheat germ, dried beet pulp, corn grits, cereal feed from corn and wheat, dried fig, soy bean oil meal, molasses, riboflavin supplement, brewer's dried yeast, Vit. A and D feeding oils, one per cent steamed bone meal, and one per cent iodized salt. According to the manufacturer, an average sample contains 23.0% protein, 6.0% fat, 9.6% ash, and 6.0% crude fiber. In addition, an average sample contains carotene, 9 mg, thiamin, 5.6 mg, and riboflavin, 5.0 mg per 100 g. Pro-cino-Rossi brand elbow spaghetti is made of "high grade A-No. 1 semolina." Analysis by the Wisconsin Alumni Research Foundation demonstrated that 100 g of this spaghetti contained 10.9% moisture, 0.65% ash, 0.17% ether-extractable material, 13.38% protein, 0.24% crude fiber and 74.66% carbohydrate. There were 2.25 mg of cystine, 1.0 mg of methionine and 1.84 mg of choline per gram of spaghetti. This spaghetti is not fortified with vitamins. Analysis of the Parma California Burgundy was kindly provided by Dr. Aram Ohanesian, chief chemist of Cella Vineyards. One hundred ml contained 13.2% alcohol (by volume), 0.74 g of acid (as tar-

* This work would not have been possible without the assistance of Miss Joan E. Colopy, Mrs. Velma H. Axelrod and Mrs. Rosemary Shaft.

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TABLE I. Influence of Diet on Development of Hepatic Lesions in Rats.

Diet	Total animals	Rats sacrificed when free of symptoms	Rats which died or sacrificed when moribund	Hepatic lesions
A. Purina dog chow & water	12	12 at 158 to 343 days	0	Normal liver
B. Spaghetti, olive oil & wine	45	6 at 45 to 223 days	39 at 13 to 223 days	Necrosis, fatty infiltration and fibrosis
C. Spaghetti, olive oil & 10% glucose in water	12	4 at 139 to 343 days	8 at 191 to 273 days	Fatty infiltration and fibrosis
D. Spaghetti, olive oil, wine & vitamins	12	10 at 139 or 343 days	2 at 271 and 343 days	Normal liver

taric acid), 0.14 g of reducing sugar, 0.19 g of tannins and 0.025 g of protein. The pH of the undiluted wine was 3.65. The wine was diluted with an equal volume of water and was fed in water bottles. No analysis was available of the olive oil used, but pure olive oil is said to be composed entirely of lipids (2) and is devoid of choline(3). Olive oil is notably rich in unsaturated fats; the oil used at no time appeared to be rancid. The vitamin supplement in Diet D consisted of 1 ml of an aqueous suspension containing 125 μ g of thiamine, 100 μ g of riboflavin, 125 μ g of pyridoxine and 750 μ g of calcium pantothenate per ml added to each 100 ml of diluted wine. In addition, one drop of cod liver oil USP was added to the spaghetti once weekly.

Results. The effect of the various diets tested is summarized in Table I. Diet A (Purina dog chow and tap water). These animals grow rapidly and remained healthy in appearance and behavior. All were sacrificed while in good condition from 158 to 343 days after the onset of the experimental period. Autopsy showed no significant abnormality. Histologically the liver and kidney were normal in every instance.

Diet B (spaghetti, olive oil, and wine). The rats gained weight at a progressively diminishing rate for about 30 days and then gradually lost weight until they died or were sacrificed. Six rats were sacrificed without obvious symptoms from 45 to 223 days after the start of the experiment, while 39 died or were sacrificed when moribund at periods ranging from 18 to 223 days.

Seven of the 45 animals showed severe dif-

fuse hepatic necrosis. This lesion was noted in 4 of the 12 rats which died within 115 days of the start of the experiment and in only 3 of the 33 rats which survived longer than this. The necrosis involved all lobules and affected small groups of cells. The latter were usually shrunken and presented acidophilic homogenous cytoplasm with indistinct borders, while the nuclei were reduced in size, pyknotic, and often fragmented. There was also focal coagulation necrosis with no nuclear content. The necrotic cell groups alternated with fairly well preserved hepatic cells, whose cytoplasm was vacuolated. Marked fatty degeneration was present in all the necrotic livers. No leukocytic reaction was evident. The liver cords were commonly fragmented and the sinusoids prominent. In a few animals there were foci of necrosis in which there was complete loss of liver architecture. These lesions were associated either with hemorrhage or hyperemia. There were no distinct nodular regenerative foci. There was slight to moderate fibrosis, bile duct proliferation, and lymphocytic exudate in the portal areas. However the fibrous reaction within the lobules was mainly of condensation type. In 8 additional rats on this diet there were slight to moderate grades of fatty degeneration or fatty infiltration, mainly periportal in location and in a few instances associated with a mild degree of fibrosis.

Diet C (spaghetti, olive oil, and 10% glucose in water). These rats gained a small amount of weight for a month or 2, and then their weight either remained relatively stationary or decreased slightly until they died or were sacrificed. Four animals in this group

were sacrificed without symptoms at 139 to 143 days and 8 died or were sacrificed in a moribund state at 139 to 273 days. In the rats in this group, parenchymal necrosis was not observed. However there were 5 animals with moderate or marked fatty infiltration of the liver, principally periportal, with formation of the signet-ring-type of hepatic cell. All were associated with a mild degree of periportal fibrosis.

Diet D (spaghetti, olive oil, wine, and vitamins). These rats either gained weight slowly but steadily or reached a maximum from which their weight did not decline, unlike the rats fed Diets B and C. Ten rats were sacrificed without symptoms at 139 to 343 days, one died at 271 days, and one was sacrificed when moribund at 343 days. There were no significant lesions of the liver in this group. No hepatic cell necrosis was found. There was one animal with a slight degree of periportal fat infiltration.

During the first 60-day period, at a time when none of the rats on measured diets had died or been sacrificed, a group of 10 rats fed diet B ate an average of 9.6 g of spaghetti and drank 14.1 ml of diluted wine per day; spillage prevented an accurate measure of the olive oil ingested. Rats offered Diet C averaged 7.9 g of spaghetti and 19.4 ml of glucose daily, and rats fed Diet D, 9.4 g of spaghetti and 10.8 ml of wine, as well as vitamin supplements. Thus rats eating a diet of spaghetti, olive oil and wine, with or without vitamin supplements, consumed 17 mg of choline, 18 mg of methionine, and 21 mg of cystine daily. Those fed a diet in which glucose was substituted for wine consumed 14 mg of choline, 15 mg of methionine, and 18 mg of cystine daily. It cannot be assumed, however, that the rats continued to ingest these quantities of food at a later time.

Discussion. Chronic alcoholism is common in patients with Laennec's cirrhosis of the liver in the United States. Nonetheless, it has not been possible to demonstrate that the administration of alcohol to experimental animals leads to hepatic lesions. Within recent years, attention has been drawn to the possible part played by chronic dietary de-

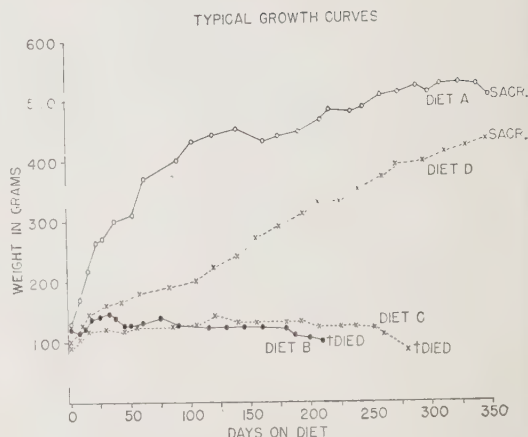


FIG. 1. Typical growth curves of rats fed Diets A, B, C and D.

ficiency in the pathogenesis of cirrhosis in chronic alcoholic patients. This view has been supported by the apparent beneficial effects of a nutritious diet in patients with this disease and by the production of hepatic lesions in experimental animals. In general, the lesions in experimental animals have been of two types, namely, fatty infiltration and fibrosis, prevented by choline or methionine, and necrosis of liver cells, prevented by cystine or methionine(4-6). However, the diets used in most experimental studies have been composed of foods not ordinarily eaten by human beings.

In the present experiments, rats were fed diets containing foods commonly used by man. These diets consisted of some of the foodstuffs consumed by patients with cirrhosis who are of Italian extraction, an ethnic group in which this disease occurs more commonly than in the rest of the population of the United States(7). In these experiments, both diffuse necrosis of liver cells and fatty infiltration and fibrosis were noted; the relative frequency of the lesion depending upon the nature of the diet and the duration of survival. Rats fed spaghetti, olive oil and wine (Diet B) developed necrosis of liver cells, fatty degeneration and fibrosis. Necrosis was relatively more frequent in those rats which died within the first 4 months of the experiment than in those which survived longer than this time. Rats fed spaghetti, olive oil and wine, supplemented by a vita-

min mixture not containing tocopherol (Diet D) had no significant lesions even though they did not appear to ingest more spaghetti than the rats fed the same diet without vitamins. The substitution of glucose for wine, without the addition of vitamins (Diet C) somewhat prolonged the time of survival, and at the same time modified the nature of the lesion so that fatty infiltration and fibrosis developed rather than necrosis. It is noteworthy that the glucose-fed rats obtained relatively more of their calories from glucose and less from spaghetti, when compared to wine-fed rats. The spaghetti was the sole source of choline and methionine.

Summary. Significant hepatic lesions, consisting of diffuse parenchymal necrosis and marked fatty infiltration, have been produced in rats fed diets of spaghetti, olive oil and

wine or glucose solution. Both lesions were associated with a moderate degree of fibrosis. The specific dietary factors responsible for the changes were not ascertained.

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Lipides Distribution and Phospholipide Turnover in Tissues of Rabbit Shortly After Growth Hormone. (22117)

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The concept that arteriosclerosis is a disease of metabolism has focused attention on endocrine control of lipid metabolism and their relationship to development of arterial degenerative lesions. It is increasingly evident that for elucidation of this relationship more information is required of the modes of action and nature of effects of hormones on lipid metabolism in different tissues. Certain adrenocortical steroids(1-3) and epinephrine (3-5) were reported to exert an influence on lipid distribution in tissues and development of aortic degenerative changes. There is ample evidence that growth hormone causes accelerated mobilization and catabolism of endogenous fat(6-10), but there is little information concerning the effect of growth hormone on lipid partition in tissues. Recently, Greenbaum and MacLean(7) studied distribution of lipides in plasma and liver of intact rats at intervals after treatment with growth hormone. Greatest changes occurred

6 hours after growth hormone when plasma and liver neutral fat and plasma phospholipide were significantly increased. The authors suggested that increased plasma phospholipide could be explained by accelerated synthesis of this lipid since Geschwind *et al.*(11) reported increased turnover of liver phospholipide in normal and hypophysectomized rats after 14 days or more of treatment with growth hormone. Mayer and Jones(12) reported a significant, but slight, increase in blood cholesterol of mice after 2 weeks treatment with growth hormone.

The present study was undertaken to determine concurrently lipid partition and phospholipide turnover in plasma, liver and aorta of intact rabbits a few hours after treatment with growth hormone. Knowledge of phospholipide-cholesterol-neutral fat relationships and phospholipide metabolism in the above tissues was a necessary preliminary to a study of the influence of growth hormone on lipid

TABLE I. Summarized Data of the Effect of Growth Hormone on Body and Organ Weights, Plasma Glucose, Liver Glycogen.

	Plasma glucose, mg %	Liver glycogen, g %	Liver wt, g/kg	Aorta wt, g	Body wt, kg
Controls (9)	118 $\pm$ 1.7*	3.44 $\pm$.35	29.0 $\pm$ 1.2	.409 (.300-.472)†	2.4 (1.9-2.7)
G.H. (9)	132 $\pm$ 2.3	4.43 $\pm$.19	35.5 $\pm$ 1.2	.404 (.346-.466)	2.4 (2.2-2.7)
P =	<.001	.02	.004	ns†	ns

* Mean $\pm$ S.E.

† Mean and range of values in group.

‡ ns = not significant.

"P" = Probability of difference between means occurring by chance alone.

metabolism in experimental atherosclerotic rabbits.

Materials and methods. New Zealand white female rabbits of pedigreed stock were given a rabbit pellet diet and tap water *ad libitum* for several weeks prior to day of experiment. Food and water were not available during experimental period. Control animals received a single subcutaneous injection of physiological saline (0.5 ml/kg body weight). Another group of rabbits received a single subcutaneous injection of 5 mg/kg of anterior pituitary purified growth hormone (Armour Co., Lot #M308)* in sterile saline 10 mg/ml. Immediately after injection of the placebo or growth hormone, 0.5 mc of radioactive phosphate (P-32)† was given intravenously. Sample of heart blood (4-5 ml) was obtained 3 hours after P-32 injection for determination of radioactive and chemical plasma acid-soluble phosphates(13). Six hours after initial hormone or saline injection a large sample (30-40 ml) of cardiac blood was obtained, the animal then killed with intravenously administered overdose of EVIPAL (n-methyl-cyclo-hexenyl-methylbarbituric acid). Whole liver and aorta were removed. A section of liver (approx. 0.5 g) was taken for determination of liver glycogen(14,15). Plasma glucose was determined by the method of Nelson(16). A section of liver (about 2 g) was placed in ice-cold 10% trichloroacetic acid and used for determination of inorganic and organic acid-soluble phosphates(17). Following homogenization of the liver sample, neutralization of filtrate to phenol red, and

addition of magnesia mixture the samples were kept in the refrigerator overnight to permit magnesium ammonium phosphate to settle (18). After centrifugation determinations were done for P-31 and P-32 on the supernatant (organic acid-soluble phosphates) and the acid-dissolved precipitate (inorganic acid-soluble phosphates). A large specimen of liver, the whole aorta, and a plasma aliquot of terminal blood sample were submitted to hot alcohol-ethyl ether extractions. Combined alcohol-ether extractions were evaporated under reduced pressure, and residue redissolved in petroleum ether (b.p. 30-60°C). Suitable aliquots of petroleum ether were used for chemical and radioactive determinations as follows: Total lipid was calculated from weight of residue after evaporation of petroleum ether and drying to constant weight. Lipide phosphorus was determined(13,19), and phospholipides were calculated from lipid-P values $\times$ 26. Total and free cholesterol were determined by the method of Schoenheimer and Sperry(20). Neutral fat was calculated by subtraction of phospholipide, free cholesterol, and cholesterol oleate concentrations from total fat value. For radioactivity determinations of phospholipide and acid-soluble phosphorus suitable aliquots were mounted on Whatman ash-free filter paper backed by aluminum foil, and samples allowed to evaporate. After covering samples with Scotch tape, they were counted with a Victoreen No. 1B85 Geiger tube for a minimum of 4000 counts.

Results. Inspection of the data in Table I shows that plasma glucose and liver glycogen concentrations, and total liver weight were moderately, but significantly, increased a few hours after growth hormone.

With the exception of liver ester cholest-

* The growth hormone used was supplied through the generosity of Dr. Irby Bunding of Armour and Co.

† Obtained from Abbott Laboratories, Oak Ridge, Tenn. as sodium radio-phosphate, sterile solution.

TABLE II. Effect of Growth Hormone on Concentration of Lipide Fractions of Plasma, Liver and Aorta 6 Hours after Administration in Rabbits.

Groups	Total lipide	Phospho- lipide	Cholesterol			Neutral fat
			Total	Free	Ester	
Liver lipid partition†						
Controls (9)	5.01 ± .15*	3.76 ± .07	.27 ± .01	.24 ± .008	.03 ± .008	.96 ± .13
G.H. (9)	6.24 ± .26	3.27 ± .09	.24 ± .01	.20 ± .008	.04 ± .006	2.54 ± .28
P =	.001	<.001	.05	.002	ns	<.001
Plasma lipid partition						
Controls	.323 ± .031	.093 ± .012	.071 ± .007	.025 ± .002	.046 ± .005	.125 ± .015
G.H.	.599 ± .034	.154 ± .007	.114 ± .011	.043 ± .004	.072 ± .008	.245 ± .019
P =	<.001	<.001	.003	.001	.015	<.001
Aorta lipid partition						
Controls	8.68 ± .61	.99 ± .03	.18 ± .003	.15 ± .004	.03 ± .004	7.49 ± .61
G.H.	6.61 ± .61	.95 ± .05	.19 ± .008	.16 ± .01	.03 ± .007	5.70 ± .58
P =	.025	ns	ns	ns	ns	.045

* Mean ± S.E.

† All values expressed as g/100 g wet tissue wt or per 100 ml plasma.

"P" = Probability of difference between means occurring by chance alone.

terol, concentrations of the lipide fractions in plasma and liver were significantly changed following administration of growth hormone (Table II). Concentrations of liver phospholipide and free cholesterol were significantly decreased, while neutral fat was significantly elevated. Concentrations of all plasma lipide fractions were significantly increased after treatment with growth hormone. With regard to the aorta lipide partition, only a significantly decreased neutral fat was found in the treated group which was mainly responsible for depression of the aorta total lipide level.

In Table III are presented the specific activities of phospholipide of plasma, liver and

aorta, and specific activities of acid-soluble phosphates of liver and plasma. Liver phospholipide specific activity was significantly increased in growth hormone-treated animals. Plasma phospholipide specific activity in the experimental group was not different from controls. However, this value, *per se*, is not a valid index of plasma phospholipide turnover since there had occurred a significant increase in chemical concentration of this lipide fraction. Calculation of the amount of radioactive phospholipide per ccm of plasma (specific activity × concentration) showed that the actual amount of plasma phospholipide synthesized during experimental period was 250% greater in

TABLE III. Effect of Growth Hormone on Turnover of Lipide and Acid-Soluble Phosphorus of Liver, Plasma, and Aorta.

Groups	Liver phospholipide-P		Plasma phospholipide-P		Aorta phospholipide-P	
	mg/100 g	S.A. × 10 ³	mg/100 ml	S.A. × 10 ³	mg/100 g	S.A. × 10 ³
Controls (9)	148.7 ± 4.2*	9.5 ± .9	2.52 ± .5	3.5 ± .6	37.18 ± 2.7	5.6 ± .4
G.H. (9)	130.9 ± 2.7	13.1 ± 1.4	5.87 ± .6	3.8 ± .7	38.93 ± 2.2	6.9 ± .7
P =	ns	.05	<.01	ns†	ns	ns
Plasma						
Liver						
Liver						
Organic acid-soluble phosphate						
mg/100 g S.A. × 10 ³						
Controls	5.75 ± .3	87.8 ± 8.5	25.0 ± .9	143.2 ± 15.0	105.0 ± 2.4	46.0 ± 5.3
G.H.	5.67 ± .2	93.1 ± 11.6	23.5 ± 1.5	136.3 ± 12.4	107.0 ± 4.7	41.9 ± 3.5
P =	ns	ns	ns	ns	ns	ns

* Mean ± S.E.

† Amount of radioactive phosphorus (S.A. × conc.) incorporated in plasma phospholipides of rabbits injected with growth hormone was significantly greater than controls (P = <.001).

S.A. is specific activity expressed as percentage of injected dose/mg of phosphorus.

"P" = Probability of difference between means occurring by chance alone.

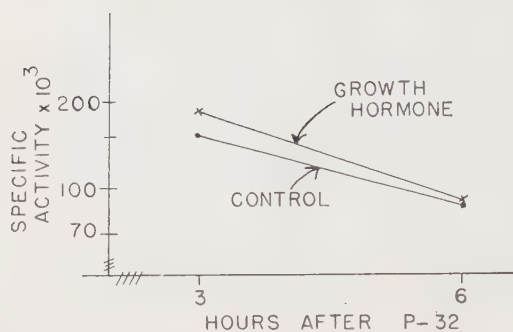


FIG. 1. Semi-logarithmic plot of the specific activities of plasma acid-soluble phosphate against time.

rabbits which received growth hormone than in controls. Inspection of terminal specific activities of acid-soluble phosphates of plasma and liver (Table III) and specific activity curves of plasma acid-soluble phosphate (Fig. 1) show that neither chemical concentration nor turnover of precursors of liver and plasma phospholipides were altered after growth hormone. It, therefore, may be concluded from the radioactivity data that accelerated formation of plasma and liver phospholipides occurred in treated rabbits. In contrast, the data show that specific activity of aortic phospholipide was not affected by growth hormone administration.

The magnitude of alterations (expressed as percentage change from controls) in various lipid fractions of plasma, and the entire liver and aorta of rabbits treated with growth hormone is shown in Fig. 2. Also shown for comparison are data recalculated from a recent experiment(5) illustrating changes in lipid fractions after a single subcutaneous injection of epinephrine in oil (1 mg/kg). Except for the different hormones administered, the conditions and experimental procedures in the two studies were exactly the same. It may be noted that in the group which received growth hormone, the greatest changes occurred in the neutral fat fraction of tissues analyzed: liver (+245%), plasma (+95%), and aorta (-25%). Also, liver ester cholesterol was changed +200%, while liver free cholesterol and phospholipide were considerably less (+25%). The plasma phospholipide, free and ester cholesterol were about 50% greater than control levels. On the other hand, 6 hours after epinephrine injection the changes

determined in lipid fractions of plasma, liver and aorta were: liver neutral fat +217%, liver ester cholesterol +100%, and plasma phospholipide +25%. The extent of increases in the two liver lipid fractions after epinephrine was comparable to that found with growth hormone. In contrast with the alterations in lipid distribution produced by the latter hormone, it is apparent that the effects of epinephrine on lipid composition in these tissues was comparatively small.

Discussion. Our data showed that marked alterations in lipid distribution of plasma, liver and aorta, and significantly increased turnover of liver and plasma phospholipides occurred 6 hours after injection of growth hormone.

Changes in amounts and rate of synthesis of phospholipides of plasma and/or liver have been reported after exogenous administration of a variety of hormones and after destruction of function of certain endocrine gland(s). In studies in which blood cholesterol also was determined, a parallelism was noted in incre-

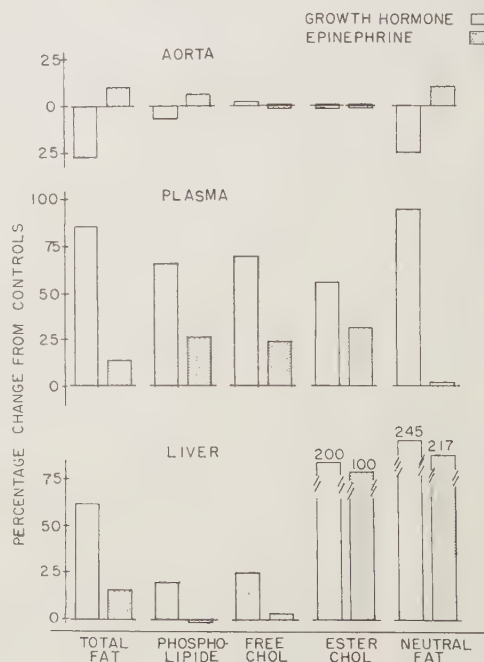


FIG. 2. Effect of growth hormone and epinephrine (see text for doses) on total lipid and lipid fractions in plasma and entire liver and aorta 6 hr after injection. Data are expressed as percentage change, increase or decrease, from respective control values.

ment or decrement of phospholipide and cholesterol levels(1-3,21). In this investigation also blood phospholipide and cholesterol were found elevated to a similar extent and accompanied by increases in the entire liver content of these two lipides. It is possible that related metabolic mechanism(s) are responsible for the regulation of blood levels of phospholipide and cholesterol; this control probably is hormonal. The radioactivity data indicated that accelerated synthesis of liver and plasma phospholipides occurred in animals administered growth hormone. Further, since practically all plasma phospholipide is believed to be produced in the liver the increased content of plasma phospholipide during the period of the experiment suggests an increased exchange of this lipid between liver and plasma had occurred. It does not seem feasible, however, at this time to deduce that alteration in rate of formation or amounts of phospholipides resulted from a specific action of growth hormone on this parameter of metabolism. This appears warranted in view of the variety of individual hormones and endocrine situations with which a change in phospholipide metabolism has been associated (22). Moreover, there is evidence(23) that changes in amount of phospholipide and their rate of formation in a given tissue are related to metabolic needs of the tissue, as an increased rate of fat catabolism. In considering this, Lotspeich and Petersen(9) and Campbell and Davidson(6) recently reported increased acetoacetate formation and oxygen uptake in liver slices obtained from rats a few hours after treatment with growth hormone. They interpreted these findings as indicating an accelerated catabolism of endogenous fat. Our data suggest that fat mobilization (accumulation of liver neutral fat and elevated plasma levels of this lipid) occurred concurrently with alterations in chemical concentrations and turnover of phospholipides of liver and blood. It, therefore, appears more likely, at least from the data now available, that increased amounts of phospholipide and their increased rate of formation a few hours after growth hormone, were not the resultant of a direct effect of this hormone on phospholipide metabolism but rather alterations in the latter

concomitant but subsidiary to the primary action of growth hormone of accelerated mobilization and catabolism of endogenous fat. It is noteworthy in this regard that Dury(5) recently reported evidence of markedly increased turnover of phospholipide of plasma, liver, and aorta in intact rabbits shortly after a single injection of epinephrine while there occurred only a moderate increase in liver neutral fat, and none in the plasma.

Change in aorta lipid distribution after growth hormone has not to our knowledge been reported. The depressed level of aortic neutral fat is especially intriguing since in other studies done at this laboratory(3-5,24) it was found that aorta lipid distribution was not altered after acute or chronic exhibition of cortisone or epinephrine. Speculation on this finding is rendered difficult in view of the paucity of information on arterial lipid metabolism, and especially the influence of hormones on the latter. Two points, however, are noteworthy as possibly related to this subject: 1) data in this and other studies have demonstrated that the aorta is particularly rich in neutral fat; and 2) aorta lipid composition was affected, at least qualitatively, as adipose tissue following administration of growth hormone.

The magnitude of alterations of lipid distribution in plasma and liver and rapidity of occurrence were remarkable. Calculation of the relative contribution of different fractions to increased total lipid in the entire liver after growth hormone revealed that neutral fat contributed 47%, phospholipide 15%, free and ester cholesterol each contributed 1.5%. With regard to total lipid increment in plasma, the relative contribution of neutral fat was 53%, phospholipide 19%, free cholesterol 6%, and ester cholesterol contributed 8%. The decreased level of aorta neutral fat was responsible entirely for the fall in aorta total lipid content. Lotspeich and Petersen (9) suggested that the liver fat mobilizing action of growth hormone might be a secondary phenomenon which occurred as a result of a primary depletion of glycogen. We did not find such a change in the intact rabbit. Indeed, in this study the liver glycogen and blood glucose concentrations were moderately,

but significantly, greater than the respective control values. Since the experiment of above authors and the present one are not exactly comparable (differences of species, alimentation, and hormone dosage) a feasible explanation cannot be made of the different effects observed on carbohydrate metabolism.

We are inclined to believe that alterations in lipides distribution and accelerated phospholipide metabolism, in this study, are best explained by the concept proposed by Weil (10) of a primary adipokinetic action of growth hormone. Since this hormone apparently modifies lipid metabolism without mediation of other target glands, further studies concerning the role of growth hormone, if any, in the patho-physiology of arteriosclerosis seem warranted.

Summary. 1. A study was made of the effects of a single injection of anterior pituitary purified growth hormone on distribution of lipides and turnover of phospholipides in plasma, liver and aorta a few hours after administration in intact rabbits. 2. Plasma glucose and liver glycogen concentration, and total liver weight were moderately, but significantly, increased in animals which received growth hormone. 3. Contents of all lipid fractions of plasma and entire liver were significantly increased in experimental animals. In the aorta, the only change was a significantly decreased neutral fat content. 4. Liver total lipid content was increased 64%, plasma total lipid was increased 85%, and aorta total lipid was decreased 25%. The relative contribution of each lipid fraction to percentage increased or decreased total lipid determined in these tissues was as follows: in liver, neutral fat contributed 47%, phospholipide 15%, and free and ester cholesterol 1.5% each; in plasma, neutral fat contributed 53%, phospholipide 19%, free cholesterol 6%, and esterified cholesterol 8%; in the aorta, neutral fat contributed 100%. 5. Radioactivity data showed that accelerated synthesis of liver and plasma phospholipides occurred after treatment with growth hormone; but aortic phospholipide turnover was not different from controls. 6. It is suggested that increased rate of formation of phospholipides of liver and plasma need not neces-

sarily be a specific effect of growth hormone but a change in this parameter of metabolism subsidiary to enhanced mobilization of endogenous fat. It is further suggested that all elements of altered distribution of lipides in tissues analyzed could be explained in accordance with the concept of a primary adipokinetic action of growth hormone.

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Comparison of Extracts of Tubercle Bacilli and BCG as Immunizing Agents in Guinea Pigs.* (22118)

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Recent investigators studying biological properties of fractions of tubercle bacilli have for the most part used the mouse for *in vivo* studies. Bloch and Noll(1) reported that tuberculosis in black mice of Strain C57 is enhanced by administration of cord factor. Weiss and Dubos(2) report that a fraction extracted from *Mycobacterium tuberculosis* by absolute methanol is capable of eliciting in mice a state of increased resistance to infection.

Notwithstanding these contributions based on a mouse evaluation of biological properties of fractions and extracts of the tubercle bacillus, we wish to report studies carried out with guinea pigs for *in vivo* determinations of immunizing activity of extracts of mycobacteria. Accordingly, we present results of experiments concerning immunizing activity in guinea pigs of extracts of non-heat killed tubercle bacilli.

Methods and materials. Most fractionation studies reported at this time were made using *Mycobacterium tuberculosis* var. *hominis* NIH strain 199RB. This culture has been used since the inception of this research in 1946.‡ Studies of strains more recently isolated from sputum are currently being made in an attempt to find a culture possessing greater immunizing potency than does 199RB. Variations were made in procedure for growth and fractionation of cultures from one experiment to another, to evaluate critically the conditions necessary

for isolation of the most potent fraction; however, procedures in general were as follows. Mass cultures were prepared on modified Long's medium(3) made up in 25 liter quantities and distributed into one-gallon jugs, each one containing 900 ml. The inoculum for 30-60 such cultures, usually a 2-week-old surface growth, was taken from one 900 ml culture flask. The cultures were subsequently incubated at 37°C for 3 to 12 weeks. After incubation, the culture fluid was withdrawn into 5 gallon bottles containing Cresylone§ and mineral oil (antifoam), autoclaved and discarded. The culture pellicle from harvested jugs was combined, by means of a vacuum system, into one container which was then completely filled with the first extracting solvent (usually ethanol) and allowed to stand 48 hours. This step can usually be considered the point at which the cells become nonviable. Following ethanol treatment, the residue was extracted 24-48 hours with ethanol-ether (50-50), methanol-chloroform (50-50), or chloroform.

Preliminary separation of the extracted components was occasionally made by cooling the methanol-chloroform solution first to 5°C and collecting a 5°C insoluble fraction. The filtrate from this separation was cooled to -20°C at which temperature a second insoluble residue was collected. The filtrate upon evaporation deposits a residue designated the -20°C soluble fraction. Further separations of total extracts or low temperature insoluble fractions were made by column chromatography. For experiments here reported, final separations were made on 50 mm diameter chromatographic columns packed with 85 g of Magnesol||-Celite¶ and charged with 2 g of

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§ Cresylone- tri-cresol disinfectant of Parke-Davis and Co.

|| Magnesol—Magnesium Silicate, Product of Westvaco Chemical Co., S. Charleston, W. Va.

¶ Celite—Filter Aid, Johns Manville Co.

the substance to be separated. The columns were developed as adsorption-elution chromatograms with the following series of solvents; petroleum-ether (60-68°C boiling range), petroleum ether-benzene (50-50) benzene, benzene-diethyl ether (50-50) and various mixtures of methanol and chloroform. Infrared spectra were recorded for all extracts and chromatograph-eluted components for purposes of identification of the substance and as a control on reproducibility of the fractionation (Randall *et al.* 4-7). Guinea pigs weighing approximately 500 g were vaccinated with the lipid fractions dissolved in a mixture of 9 parts mineral oil and one part Arlcel A.** Bacillary residues and protein extracts were emulsified from aqueous suspension in the same adjuvant according to the method of Salk(8). As one of the controls, BCG was routinely administered in aqueous suspension either from cultures grown 8 days on liquid Tween-albumin medium or from lyophilized vaccine kindly supplied by Tice Laboratories through the courtesy of Dr. Sol. R. Rosenthal. All animals were vaccinated subcutaneously in the inguinal region or over the sternum. The protocol for each evaluation of bacillary extracts *in vivo* was changed from time to time but generally guinea pigs were vaccinated with 0.1 mg of extract or 1.0 mg of BCG. Three weeks after the second vaccination test guinea pigs and a suitable number of controls, treated with adjuvant alone, were shaved and tuberculin tested with 0.0002 mg of PPD by intracutaneous route. The results were read at 24 and 48 hours, a positive reaction being at least 5 mm minimum diameter. In many instances, reactions were strong with a blanched, later necrotic, center. Following the tuberculin test, all groups were randomized and challenged. The 18-20 guinea pigs in each group were housed in 3 or 4 stainless-steel cages. For randomization, the 3 or 4 cages in any one experimental group were redistributed at random so that one cage would be found in the first quarter of the total number, a second cage in the second quarter of the total and so on. The code for the newly as-

signed cage numbers was sealed and not examined until post mortem examination of all groups had been completed. The following advantages were presumed to result from randomization. It would tend to minimize the possibility that variations in challenge suspension or procedure would affect any one experimental group, and would also reduce unintentional bias which might be shown in evaluating the extent of gross disease at autopsy. During post mortem examination, each successive series of 5 animals has come from a different treatment group. This results in a constant change in extent of gross tuberculosis and would discourage any inadvertent attempt to reduce the variation from one group to the next.

The challenge suspension was prepared from an 8-day culture of strain 199RB on liquid Tween-albumin medium. The culture was first filtered through Whatman #1 filter paper, adjusted to 85% transmission at 500 μ (Coleman Junior spectrophotometer) and then diluted to 10^{-5} with saline containing 0.5% bovine albumin fraction V. Plate counts made of this challenge suspension contained 300-1000 viable units. The challenge was given subcutaneously in 1 ml of diluent in the inguinal region or over the sternum. Six weeks after challenge the guinea pigs were sacrificed (ether) and the extent of gross tuberculosis in the viscera estimated according to the scheme of Feldman(9). It is important to state that gross evaluation of the lungs was difficult to make in early experiments because of intercurrent infection with group "C" streptococcus, however it has been our experience with strain 199RB that the spleen is a good index of the extent of tuberculosis in the guinea pig. In order for this to be true, the challenge should be such that some animals will have minimal or no gross disease. If the spleen is extensively involved, showing enlargement and confluent tubercles, one expects to find disease in the lungs and liver. Conversely, if there is no splenic involvement, little disease is seen in other organs. Observations were also made on the site of inoculation, as well as inguinal, tracheo-bronchial, and retrosternal lymph nodes. The

** Arlcel A—Product of Atlas Powder Co., Wilmington, Del.

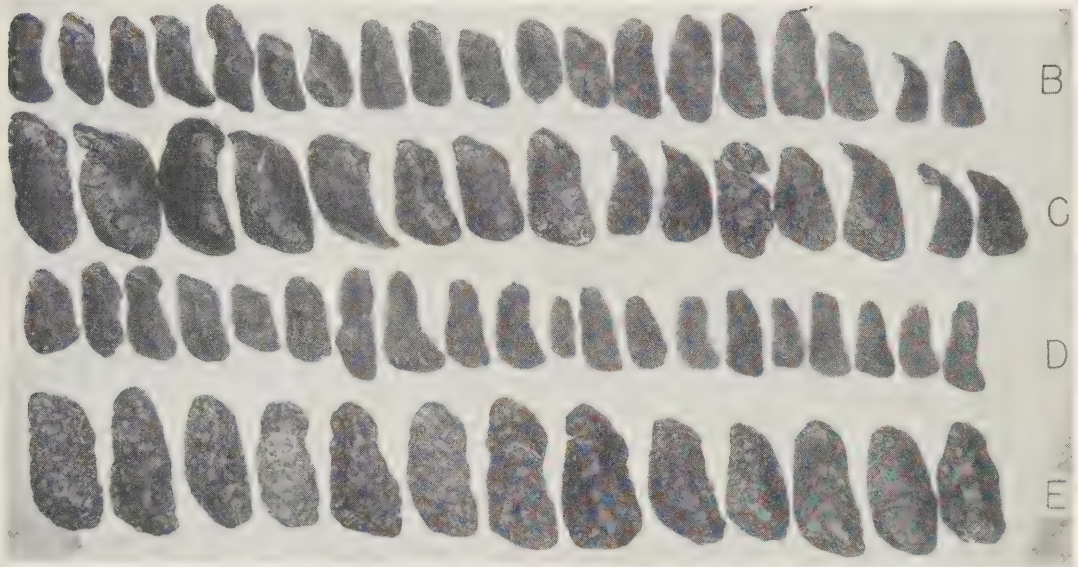


FIG. 1. Photograph of formalin fixed spleens from 4 groups of animals taken from the experiment recorded in Table IV.

- Group B—vaccinated with 0.05 mg of defatted bacilli
- " C—alcohol-ether soluble fraction
- " D—1.0 mg of lyophilized BCG
- " E—non-vaccinated controls

Spleens are arranged in approximate order of decreasing amount of gross disease reading and numbering from left to right. Typical Feldman Index values for spleen are as follows. Group B—First 4 spleens, value 20; spleens 5, 6 and 7, value 15; spleens 8 through 13, value 10; spleens 14 through 17, value 5; and spleens 18 and 19, value 0. Group C—All spleens given value of 35 with exception of last 2 which were recorded as 30 and 25 respectively. Group D—Spleen 1, value 20; spleen 2 through 5, value 15; spleens 6 and 7, value 10; spleens 8 through 12, value 5; and spleens 13 through 20, value 0. Group E—Spleens 1 through 18 recorded as 35; spleen 19 recorded as 30 (this group was reduced for photographic purposes by leaving out 6 spleens between spleens 1 and 2).

extent of gross disease was recorded according to the scheme of Feldman, slightly modified. A value of 5-10 was recorded for lungs on which less than 12 surface lesions could be counted. For greater than 12 lesions, values of 15 and 20 were assigned and when the number of lesions was too large to count, values of 25 or 30 were given depending upon the over-all appearance. By referring to Fig. 1, one can see numerical assignments typical of those made for spleen involvement.

Results. The purpose of the first experiment was examination of 3 variables, namely, age of culture to be extracted, concentration of ethanol used in the first cell treatment, and use of alpha tocopherol acetate as an antioxidant, for their effects on immunizing potency of a crude chloroform extract. Ninety bottles of modified Long's medium were inoc-

ulated with culture 199RB. Sixty were harvested after 3 weeks incubation, and the remaining 30 after 12 weeks incubation. Half of the cultures at each period were treated with 50% ethanol and half with 95% ethanol. A like division of cell mass was made for subsequent extraction with chloroform containing tocopherol or with reagent grade chloroform. This series of variables ultimately gave rise to the following eight crude lipid extracts: 1) 3W50T—extract of 3 week (3W) culture treated with 50% ethanol (50) and extracted with chloroform containing tocopherol (T), 2) 12W95—extract of 12 week culture (12W) treated with 95% ethanol (95) and extracted with chloroform, 3) 3W50, 3W95, 3W95T, 12W50, 12W95T, 12W50T.

Several observations may be made from the results of this experiment recorded in

TABLE I. Feldman Index Values for Immunized and Control Guinea Pigs Sacrificed 6 Weeks after Challenge.

Vaccine	No. of G. pigs sacrificed	Tuberculin reaction†	Mean Feldman index§
BCG*	18	—	26
3W50T†	16	+	40
3W50	16	+	40
3W95T	16	—	58
3W95	19	—	51
12W50T	16	+	44
12W50	18	+	36
12W95T	18	—	47
12W95	19	—	32¶
Control	19	—	43

* Lyophilized vaccine (Tice 968-P), 0.5 mg given twice 3 wk apart.

† All lipid fractions suspended in mineral oil containing 1 part in 20 of aquaphor (1 mg of lipid in 0.1 cc of adjuvant) in two doses 3 wk apart.

‡ Tuberculin test, 0.0002 mg of PPD intracutaneously, read at 24 and 48 hr.

§ Three wk after second vaccination all groups were randomized and challenged with 800 viable units (plate count) given in 1 cc intraperitoneally. All groups were sacrificed 6 wk after infection and extent of gross disease recorded according to the scheme of Feldman.

Autopsy index:

	Spleen	Lung	Liver
Extensive	35	30	25
Moderate	20	20	20
Slight	10	10	10

(Moderate lung 12 or more discrete tubercles, slight 2-11 tubercles given value of 10, 1 tubercle value of 5.)

|| Significant at the 1% level.

¶ " " 5% " "

Table I. There appears to be little or no immunizing value in any extract of 3-week-old cultures while at least one extract of the 12-week culture appeared to give some protection. Extracts prepared from 50% ethanol treated cultures uniformly caused the development of a high level of tuberculin hypersensitivity^{††} whereas no hypersensitivity developed in animals vaccinated with extracts from 95% ethanol treated bacilli or BCG. One extract (3W95T) appeared to lower the resistance of guinea pigs to subsequent infection with tubercle bacilli.

Extracts evaluated in the second test were

†† Selected animals from hypersensitive groups were removed before challenge and maintained in separate quarters as non-challenge controls. They were autopsied at the same time as other groups and the tissues were cultured and found to be negative with respect to acid-fast bacilli.

modifications of the three substances which showed some evidence of activity in the first experiment. Two extracts were precipitated at -20°C from methanol-chloroform yielding -20°C soluble fractions (-20S) and -20°C insoluble fractions (-20I). A third extract was chromatographed on a Magnesol-Celite column packed with 85 g of adsorbent. The column was charged with 2 g of the 12W50 extract and eluted with 1800 ml amounts of the following series of solvents: petroleum ether (PE), petroleum ether-benzene (PEB), benzene (B), benzene-ether (BE), methanol (M) and chloroform (C). The hold-back volume (volume of solvent retained by the packed column) for the column was approximately 300 ml and eluates were collected as complete hold-back volumes. Methanol eluates were divided into chloroform soluble and chloroform insoluble parts.

It was not possible to demonstrate resistance lowering activity with any preparation

TABLE II. Feldman Index Values for Immunized and Control Guinea Pigs Sacrificed Six Weeks after Challenge.

Vaccine	No. of G. pigs sacrificed	Tuberculin reaction‡	Mean Feldman index§
BCG*	12	+ —	8
12W95† (1 inj.)	17	—	27
12W95	14	—	25¶
" -20I	18	—	20¶
" -20S	14	—	33
3W95T	16	—	26
" -20S	17	—	35
" -20I	14	—	33
12W50	14	—	19
" PeB-2	17	—	29
" Pe (5-6)	8	—	36
" PeB-1, Pe-3	15	—	27
" BE-1	13	—	30
" BE-3	15	—	25¶
12W50M ₁ CHCl ₃ S	14	—	32
" CHCl ₃ I	15	—	26
Controls	18	—	36

* Fresh vaccine (Phipps strain) 1 mg given one time only with the first injection.

† Lipid fractions suspended in Bayloll-Arlacel, 0.1 mg given in 2 doses 3 wk apart.

‡ Tuberculin test, 0.0002 mg PPD intracutaneously, read at 24 and 48 hr.

§ Three wk after second vaccination all groups were randomized and challenged with 700 viable units of strain 199RB subcutaneously in the inguinal region. Sacrificed 6 wk after infection.

|| Significant at the 1% level.

¶ " " " 5% " "

TABLE III. Feldman Index Values for Immunized and Control Guinea Pigs Sacrificed Six Weeks after Challenge.

Vaccine	No. of G. pigs sacrificed	Tuberculin reaction†	Mean Feldman index§
BCG (1 mg)*	14	+	11
12W50 (1 mg)	20	+	24
" (1 mg)	19	+	15
" (2 mg)*	17	+	16
" -5I	18	±	22
" -20I	20	+	21
" -20S	15	—	32
" -column	17	—	36
" -column + defatted bacilli†	17	+	9
Defatted bacilli†	15	+	12
Controls	19	—	37

* Given one time only at the first inoculation (BCG fresh Phipps strain).

† Defatted bacilli were suspended in adjuvant so that each 0.1 cc would contain 0.05 mg of cells (dry wt). Unless stated otherwise all lipid fractions were given in adjuvant (0.1 mg in 0.1 cc).

‡ Tuberculin test, 0.0002 mg of PPD, read at 24 and 48 hr.

§ Three wk after second vaccination all groups were randomized and challenged with 800 viable units of strain 199RB in 1 cc of saline. The cultures were filtered through Whatman #1 filter paper and 10 cc syringes were used once only. The last 1 cc volume in each syringe was discarded. Six wk after infection all groups were sacrificed and the extent of gross tuberculosis recorded according to the scheme of Feldman.

in this test. From the Feldman index figures it appears that a significant level of resistance was stimulated only in groups vaccinated with BCG and to a lesser extent with 12W50 and 12W95-20I. However, no fraction separated from 12W50 by column chromatography possessed the level of activity found in the parent fraction. It is interesting to note that fraction 12W50 Pe(5-6) was later identified by infrared spectroscopy as a contaminating oil inadvertently introduced in one of the extraction steps. From this experiment it is to be concluded that an active immunizing component was not separated from 12W50 by chromatography. Among possibilities which may account for this failure are the following: to produce an immunizing effect in the guinea pig, several components separated on the column would have to be combined, or activity of the fraction is destroyed on the chromatographic column, or finally that the active material is not eluted from the column.

In the experiment in Table III a new column was prepared and the 12W50 fraction applied in the same manner. The column was developed with the same series of solvents, however, this time all eluates were collected in one receiver and evaporated to dryness. The residue collected in this manner was tested as such and also after combining it with bacillary residue remaining after neutral solvent extraction (defatted bacilli). In addition, the 12W50 fraction was precipitated at 5°C and -20°C.

Comparing the results of this experiment with the previous one, it can be seen that approximately the same level of immunizing activity was obtained with 0.1 mg amounts of the 12W50 fraction, the chloroform soluble fraction of 50% ethanol treated bacilli. When the amount of fraction injected was increased to 1 mg in two injections or 2 mg in one injection, a higher degree of resistance to infection was produced. Fractions precipitated at 5°C and -20°C appeared to contain all the activity of the parent fraction, but no increase in activity was noted. The pool of eluates from chromatographic separation of 12W50 had completely lost all the original activity, however, when mixed with defatted bacillary residue, significant increase in potency of the preparation was noted. Defatted bacilli in adjuvant produced an increased resistance which compares favorably with some of the best preparations thus far evaluated. Finally it should be noted that the most significant protection was produced in animals that had been rendered hypersensitive to the extent that they gave positive tuberculin reactions with 0.0002 mg of PPD.

The last experiment is one in which further investigations of the defatted bacilli were made. Determinations were made of the effect of heat (121°C for 20 min.), extraction with borate buffer and the effect of further extraction of the residue with alcohol-ether containing 3% HCl.

From the results, Table IV, it would appear that extraction of neutral solvent insoluble residue with alcohol-ether containing 3% HCl destroys the remaining activity of this substance, since neither the soluble portion,

Anderson's(10) "firmly bound lipids," nor the residue after this extraction, possessed any ability to increase resistance of guinea pigs to tuberculosis. In addition it appears that 0.05 mg of defatted bacilli is the minimal effective amount and that autoclaving does not greatly affect the activity of the preparation. The fraction soluble in borate buffer at pH 9.1 had an intermediate activity which might have resulted from the presence of small numbers of intact cells.

Discussion. One of the major problems encountered in an examination of biological properties of fractions of tubercle bacilli, is removal of intact bacilli from extracts and fractions, Raffel(11), Myrivic(12). Results of an earlier report of this work (Smith *et al.*) (13), in which a smaller number of animals were used, are open to question because filtration was the only method employed to remove intact bacilli from extracts. In this report, the same considerations may apply to loss of activity of the 12W50 extract following chromatography.

Results obtained after vaccinating guinea pigs with small amounts of defatted bacilli in adjuvant show that significant increase in anti-tuberculous resistance has taken place. Guinea pigs vaccinated with whole dead bacilli are not protected to the same extent. These results would imply that the vaccine is improved by removing neutral solvent soluble substances (*i.e.* Cord Factor). In another experiment, not reported here, neutral solvent soluble material of a strain other than 199RB did not significantly alter resistance of guinea pigs when tested under similar conditions.

It has been reported by Anderson(10) that after extracting tubercle bacilli with neutral organic solvents it is possible to remove 10% more lipid by treating the bacilli with alcohol-ether containing 3% HCl. When these so-called "firmly bound lipids" were removed from previously extracted bacilli, neither the soluble portion nor the residue possessed any immunizing activity. Accordingly, it would appear that rupture of lipo-protein bonds which hold the firmly-bound lipids to bacillary protein renders the resulting materials inactive with respect to their resistance in-

ducing properties. It is also interesting to note that at this same stage of fractionation, the property of inducing a state of delayed hypersensitivity was also destroyed.

The fact that purified protein or lipid fractions have the ability to stimulate increased resistance to tuberculosis in mice and guinea pigs is shown by the work of Weiss and Dubos(2), Kropp and Floyd(14), Choucroun(15), and Youmans(16). The present work neither confirms nor refutes these earlier observations.

With limitations on space and animal facilities, it has been necessary to carry out evaluations *in vivo* on a screening basis using minimal numbers of guinea pigs for any one vaccine and a challenge-sacrifice type of experiment. Estimations of the extent of disease at autopsy were made according to the method of Feldman. Statistical studies by analysis of variance technic,^{††} indicates that, for groups of 14-20 animals, 10 points on the Feldman scale represents a difference which is significant at the 5% level. Since the work is still in a preliminary phase it was felt that only limited microscopic examination of tissues could be made. However, a definite correlation was shown to exist between gross and microscopic appearance of spleens and the extent of gross disease recorded for animals in several groups (BCG, 12W50 and Controls) of the experiment recorded in Table II.

Finally attention should be drawn to results obtained with BCG vaccines in the four experiments reported in this paper. Using approximately 1.0 mg of fresh vaccine (Phipps strain) or lyophilized vaccine (Tice strain), significant protection was afforded by BCG in each test. This is perhaps best shown in Fig. 1 where the spleens of four groups of the experiment recorded in Table IV are arranged in the approximate order of decreasing extent of gross disease reading left to right.

Summary. 1. Comparison has been made in guinea pigs of ability of BCG and various extracts and fractions of the tubercle bacillus to stimulate increased resistance to tuber-

^{††} Courtesy of the Numerical Analysis Laboratory, University of Wisconsin.

TABLE IV. Feldman Index Values for Immunized and Control Guinea Pigs Sacrificed Six Weeks after Challenge.

Vaccine	No. of G. pigs sacrificed	Tuberculin reaction§	Mean Feldman index
Defatted bacilli	20	+	36
.01 mg			
<i>Idem</i> .05 mg	19	+	17
" 1.0 mg*	18	+	29
DFB heated to 121°C 20 min.	17	+	27
DFB extracted (soluble) in Alc-ether (3% HCl)	17	—	62
Residue of above	20	+ —	51
Whole cells heated at 121°C 20 min.	19	+	36
DFB extracted with borate buffer	17	+ (min)	34
BCG*	19	+	11
Controls	19	—	63

* Given one time only at time of first inoculation.

† BCG given one time only 6 wk before challenge. 1 mg of 949BLP. (Lyophilized vaccine, Tice laboratories.)

‡ All fractions given as 0.05 mg in adjuvant unless otherwise indicated.

§ Tuberculin tested with 0.0002 mg of PPD.

|| All animals infected with 300 viable units of culture 199RB in 1 cc of diluent subcutaneously over the thorax. Undiluted culture was first filtered through Whatman #1 filter paper. To reduce possible variations in challenge inoculum, 10 cc syringes were used only once and the last 1 cc volume of suspension in each syringe was not used.

defatted bacilli was completely destroyed by extraction with alcohol-ether containing 3% HCl and only partially by autoclaving for 20 minutes. The ability of defatted bacilli to induce delayed hypersensitivity was also destroyed by removal of firmly bound lipids with alcohol-ether containing HCl.

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culosis. 2. Significant resistance was induced by 1 mg of BCG, given subcutaneously, in each of 4 experiments reported. 3. Comparable resistance was induced by 0.1 mg (in adjuvant) of a crude chloroform extract, and also by tubercle bacilli which had been exhaustively extracted with neutral organic solvents. 4. The immunizing potency of such

Effect of Prolonged Oral Administration of Reserpine (Reserpoid®) on Gastric Secretion.* (22119)

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An increasing number of reports in medical literature testify to the effectiveness of *Rauwolfia serpentina* Bentham and its alkaloid, reserpine, in the treatment of hypertension, psychoses, neuroses and other conditions in which anxieties, tension and aggressions play a prominent role either in symptomatology or etiology. Reserpine might also be useful in the treatment of certain gastrointestinal disorders, such as peptic ulcer, functional bowel distress, including irritable colon syndrome with diarrhea or constipation, and other types of functional digestive disturbances. The work of Bein(1,2) indicates that reserpine acts upon the central autonomic mechanisms and that this action can be explained by a direct depressant effect on the hypothalamus. This produces peripheral effects similar to those resulting from stimulation of the parasympathetic nervous system. For example, Bein(3) and Plummer *et al.*(4) observed increased motor activity of intestinal tract in animals after administration of reserpine. More important, Plummer *et al.*(4) found that an increase in both volume and hydrochloric acid content of the gastric juice of dogs followed intravenous administration of 15 mg/kg of body weight of reserpine. This increased secretion was effectively prevented for several hours by parenteral use of an anticholinergic drug. Beman(5) stated that both oral and intravenous preparations of reserpine have a potent effect on parietal cells of gastric glands in man, as shown by a significant elevation of gastric acidity, not altered by administration of atropine.

Since it is recognized that free hydrochloric acid is one of the essential factors in production of peptic ulcer and that increased gastric acidity is more common in patients with duodenal ulcer than in normal subjects, it is

of importance to determine whether prolonged oral administration of reserpine in therapeutic doses produces significant alterations in gastric acidity. It is the purpose of this paper to report effects of daily administration of reserpine orally on gastric secretion of 20 patients with various gastrointestinal disorders, including peptic ulcer.

Materials and methods. Twenty unselected patients from the Gastrointestinal Clinic, diagnosed as having duodenal ulcer, gastric ulcer, functional bowel distress, etc. (Table I), were given 0.25 mg reserpine† orally 4 times daily for 7 to 210 days; 8 were males and 12 females; age ranged 29 to 73 years. Patients with peptic ulcer were maintained on a modified Sippy diet with antacid medication. They received no anticholinergic drugs or other agents known to affect gastric secretion during this study. A one-hour basal gastric analysis was performed on each patient prior to therapy. The technic has been described previously(6). It consists first of collection via a Rehfuess tube of all gastric juice in the stomach after an over-night fast. This is called the residual specimen (R). Continuous aspiration is then carried out by means of hand syringe for 4 consecutive 15-minute periods. The (R) and each 15-minute collection (B1, B2, B3, B4) are titrated for free hydrochloric acid; total volume is measured and output of hydrochloric acid calculated. After the control basal gastric analyses were made, patients were given 0.25 mg of reserpine orally 4 times a day for variable lengths of time, and a second basal gastric analysis obtained. The results were compared with corresponding pre-treatment values.

Results. As shown in Table I, maximum free gastric acidity rose in 6 instances, fell in 13 instances, and was unchanged in one. Patients

* This study was supported by grant from The Upjohn Co., Kalamazoo, Mich.

† Supplied as Reserpoid® tablets by The Upjohn Co., Kalamazoo, Mich.

TABLE I. Individual Comparisons of Basal Gastric Analyses before and after Reserpine Therapy in 20 Patients.

Patient, sex, age * and diagnosis	Before reserpine					No. days on drug	During reserpine				
	Res.	B1	B2	B3	B4'		Res.	B1	B2	B3	B4
P.P. ♀ 72 Duodenal ulcer	HCl* Vol.†	13 10	20 24	34 20	23 17	16 14	7 8	18 8	52 7	74 3	45 6
J.S. ♂ 73 Hiatus hernia	HCl Vol.	0 32	19 13	38 11	30 3	8 8	9 4	0 15	20 14	8 5	0 8
K.B. ♀ 52 Gastric ulcer	HCl Vol.	14 17	0 10	2 8	1 9	0 7	14 57	2 16	0 13	21 10	27 14
M.H. ♂ 70 Duodenal ulcer	HCl Vol.	4 10	42 5	44 7	44 5	36 4	14 14	19 7	40 5	20 8	26 19
W.M. ♀ 56 FBD‡	HCl Vol.	60 58	43 40	70 37	85 28	85 33	14 15	34 70	75 23	72 23	63 23
J.D. ♂ 54 Duodenal ulcer	HCl Vol.	1 26	0 24	7 16	10 10	7 14	17 26	0 30	0 23	0 27	0 12
S.C. ♂ 40 FBD	HCl Vol.	18 49	21 20	40 12	10 20	4 20	24 30	12 16	18 23	26 12	38 20
D.A. ♀ 62 FBD	HCl Vol.	0 24	26 24	17 16	17 23	16 20	28 32	0 30	30 15	28 13	30 18
P.Mc. ♀ 71 FBD	HCl Vol.	0 6	0 2	0 12	0 13	4 11	31 69	0 34	0 17	3 21	0 11
E.B. ♀ 54 FBD	HCl Vol.	0 34	9 16	19 15	48 11	48 15	31 9	0 16	0 8	6 8	10 5
R.M. ♀ 63 FBD	HCl Vol.	0 2	35 9	50 5	70 10	45 3	35 16	0 7	2 12	37 8	56 13
G.G. ♂ 60 Gastric resection	HCl Vol.	25 4	20 10	25 20	35 22	40 20	37 5	0 18	0 11	0 13	12 13
P.E. ♂ 29 Gastric resection	HCl Vol.	0 14	6 8	24 7	18 6	26 6	42 10	0 5	0 3	0 9	0 9
A.D. ♂ 57 FBD	HCl Vol.	0 14	41 26	65 23	47 23	84 13	42 9	48 11	67 27	69 49	70 23
M.S. ♀ 43 Hiatus hernia	HCl Vol.	8 24	0 38	0 16	0 15	0 18	46 24	10 30	0 32	18 21	15 22
C.G. ♂ 67 Duodenal ulcer	HCl Vol.	0 10	14 6	5 6	0 6	3 4	80 10	11 8	5 7	9 7	6 3
M.A. ♀ 65 FBD	HCl Vol.	17 20	30 25	28 32	32 18	30 43	175 6	0 17	19 10	19 19	22 16
M.O. ♀ 65 FBD	HCl Vol.	15 27	24 15	18 17	17 30	26 25	210 14	0 15	45 12	26 5	14 5
M.Me. ♀ 63 FBD	HCl Vol.	0 33	12 22	20 17	12 8	15 5	52 21	0 43	0 28	0 25	20 22
O.W. ♀ 46 Pylorospasm	HCl Vol.	16 40	0 10	15 28	0 12	13 47	25 42	10 17	15 15	6 15	9 12

* Conc. in mEq/L.

† In ml.

‡ FBD = Functional bowel distress.

with peptic ulcer did not appear to react differently from those with other digestive disturbances or diseases. It is apparent that reserpine does not produce a consistent gastric secretory change in either direction. The average volume, free acidity and output of hydrochloric acid of residual specimens and of the four 15-minute basal specimens during reserpine administration showed no significant

change from control values.

None of these patients experienced any significant side effects. An occasional patient complained of a slight "stuffy" feeling in the nose.

Discussion. Prior to clinical trial of reserpine in peptic ulcer and other gastrointestinal disorders, we investigated the effect on gastric acidity of prolonged oral administra-

tion of the drug in average therapeutic amounts. Although animal experiments, theoretical considerations, and one report on human volunteers have indicated that reserpine can increase gastric acidity, under conditions of this study, no significant change of volume, free hydrochloric acid or output of hydrochloric acid was observed during reserpine administration.

It is possible that large doses of reserpine taken orally or parenterally over a short period of time may increase gastric acidity, since they have been shown to cause other severe side effects, as indicated by Noce *et al.* (7). It is also possible, however, that tranquilizing effect of well-tolerated doses of reserpine may bring about a secondary decrease in gastric acidity and may therefore be helpful in the management of the peptic ulcer patient.

Conclusions. 1. Basal gastric analyses were made in 20 patients with various gastrointes-

tinal disorders, including peptic ulcer, before and during therapy with reserpine. 2. No consistent change in volume, free hydrochloric acid concentration or output of hydrochloric acid was observed after prolonged oral administration of 0.25 mg of reserpine 4 times daily.

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Effects of Liver Fractions on Thyroid Function of the Rat.*† (22120)

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Several investigators have reported that diets containing liver produced thyroid enlargement in different laboratory animals (1-5). Hou(5) was able to demonstrate that goitrogenic activity was present in the ethanol soluble portion of ox liver. Although substantial thyroid enlargement was obtained in the latter investigation, a search of the literature has failed to yield any further information concerning the substance(s) responsible for the goitrogenicity. Anselmino and Hoffman(6) found fetal blood and other animal tissues to contain an "antithyroid protective" substance capable of overcoming the increase in basal metabolic rate caused by thyroxine injection. More recently Ershoff

(7-10) has studied the "antithyrototoxic" effect of liver in overcoming the growth retardation of rats fed thyroidactive materials.

The present investigation is part of a study designed to identify the substance(s) responsible for the goitrogenicity of liver and to determine whether its goitrogenicity is related to the "antithyroid protective" substance or the "antithyrototoxic" factor.

Experimental. Sixty male and 60 female rats of the Wistar strain (housed in individual wire cages) were divided equally into 12 groups of 10 each and treated as indicated in Table I. Pork liver fractions† were incorporated into an iodine deficient ration (Table II) and into the same ration containing adequate iodine. The liver fractions replaced

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‡ Desiccated pork liver and the water soluble and water insoluble fractions of pork liver were purchased from Wilson Laboratories, Chicago, Ill.

TABLE I. Effect of Liver Feeding on Thyroid Function.

Treatment	Iodine content of ration, p.p.b.	Thyroid wt /100 g body wt, mg*	Thyroid-iodine content†		Protein bound plasma iodine/100 ml, µg	Avg weekly gain, g
			Total, %	Protein bound, %		
Basal ration	58	7.44 ± 1.21	.0106 ± .0025	.0103 ± .0025	1.40	29.2
3% H ₂ O soluble liver	"	8.11 ± 1.36	.0133 ± .0032	.0117 ± .0032	1.62	29.2
6% " " "	"	8.52 ± 1.08	.0094 ± .0025	.0087 ± .0018	1.14	26.8
20% H ₂ O insoluble liver	"	12.28 ± 1.42	.0040 ± .0004	.0029 ± .0008	1.20	28.6
20% desiccated liver	"	12.15 ± 2.15	.0038 ± .0028	.0025 ± .0014	1.62	14.6
.01% thiouracil	"	15.83 ± 2.59	.0022 ± .0009	.0013 ± .0007	1.21	27.3
Basal ration	400	5.77 ± 1.49	.0545 ± .0080	.0463 ± .0148	1.77	28.2
3% H ₂ O soluble liver	"	5.52 ± .76	.0616 ± .0115	.0579 ± .0120	2.45	29.8
6% " " "	"	6.44 ± 1.47	.0576 ± .0120	.0567 ± .0179	2.71	26.2
20% H ₂ O insoluble liver	"	6.58 ± .70	.0639 ± .0307	.0541 ± .0295	2.70	28.8
20% desiccated liver	"	6.39 ± 1.01	.0615 ± .0291	.0594 ± .0288	3.02	16.2
.01% thiouracil	"	10.39 ± 1.30	.0096 ± .0029	.0082 ± .0023	1.89	27.4

* Mean ± stand. dev., 10 animals/group.

† Mean ± stand. dev., from analyses of thyroids from 4 animals/group.

wheat germ in the diet and the percentage composition of the diets was adjusted by varying the quantity of corn and wheat germ until each contained the same percent protein. Diets containing 0.01% thiouracil were used to compare the goitrogenic activity of the liver fractions with that of a known goitrogen. After the animals had been on the experimental diets for 39 or 40 days they were starved for 20 hours after which basal metabolic rates were determined using a modified Haldane apparatus. On the 42nd

day blood samples were taken and pooled according to treatment. Thyroid glands were excised, weighed and reserved for analysis. Protein bound plasma iodine determinations were made using the procedure of Barker (11). The following determinations were made on thyroid samples: total fresh weight, dry matter content, total iodine content, and protein bound iodine content.

Results. The goitrogenic activity of pork liver was found to be present in the water insoluble fraction and, with rats fed low iodine diets, was of nearly the same magnitude as that produced by 0.01% thiouracil. When the iodine intake was adequate the goitrogenicity of water insoluble liver was completely overcome. Desiccated liver exhibited the same degree of goitrogenicity as water insoluble liver and rats fed the former grew poorly. The concentration of iodine in the thyroid glands of rats fed the goitrogenic liver fractions and low dietary iodine was approximately one-third that of the corresponding controls. This decrease appeared to be due to lowered protein bound thyroid iodine. However, when adequate iodine was included in the diet the thyroid iodine of animals fed the goitrogenic liver fractions was the same as, or slightly higher than, that of the control animals fed the same level of iodine. Thiouracil reduced thyroid iodine concentration at both dietary iodine levels. Histological examination of thyroid sections indicated that

TABLE II. Composition of the Basal Diet.*

Ingredient	%
Corn	58.6
Wheat germ†	35.0
Cottonseed oil	2.0
DL methionine	.4
Mineral mixture‡	4.0

* The following amounts of vitamins were added to this diet (expressed in mg/100 g): riboflavin 1, thiamine hydrochloride 0.75, niacin 5, *i* inositol 30, pyridoxine hydrochloride 1, choline chloride 200, biotin 0.01, folic acid 0.01, calcium pantothenate 5, *p*-amino benzoic acid 30, vit. B₁₂ 0.002, 2-methylnaphthoquinone 0.50, α -tocopherol 10, vit. A acetate 0.05, and calciferol 0.005.

† Wheat germ which had been partially defatted, dehydrated and pulverized was purchased from Viobin Corp., Monticello, Ill.

‡ The mineral mixture had the following percentage composition: sodium chloride 4.43, magnesium sulfate (anhydrous) 6.78, disodium phosphate 8.86, monopotassium phosphate 19.03, potassium chloride 10.40, calcium phosphate monobasic 13.75, calcium lactate 33.07, ferrous ammonium sulfate 3.63, manganese sulfate 0.016, copper sulfate 0.024, and zinc carbonate 0.024.

water insoluble liver was as active as 0.01 percent thiouracil in causing loss of colloid, and epithelial hypertrophy and hyperplasia. Slight variation was observed in protein-bound plasma iodine values at the low dietary iodine level, but adequate dietary iodine resulted in increased plasma protein bound iodine values with rats fed all the liver fractions. This was not accompanied by an increase in basal metabolic rate. No significant differences in basal metabolic rate were observed between treatments when expressed either as a function of body weight or of body surface area.

Discussion. Rats fed low dietary iodine and water insoluble liver had greatly reduced protein bound thyroid iodine stores but when the liver fed animals received adequate dietary iodine not only did thyroid protein bound iodine return to normal but plasma protein bound iodine values were nearly doubled. This appears to indicate an increased synthesis and secretion of thyroxine rather than a decreased rate of thyroxine synthesis as is observed with thiouracil. The effects of the liver fractions on thyroid size and the level of protein bound iodine in the thyroid and in blood plasma seem to be dependent upon the level of iodine available to the thyroid. It would also appear that whatever causes this effect on thyroid and plasma protein bound iodine is either partially water soluble or different than the agent responsible for the thyroid enlargement observed in rats fed water

insoluble or desiccated liver. In either case it appears that the mode of action of the goitrogenic factor(s) in liver is unlike that of thiouracil.

Summary. The present study is part of an investigation initiated in order further to purify and evaluate the goitrogenic substance (s) present in liver. The activity was found to be in the water insoluble portion of pork liver. Increased dietary iodine completely overcame its effect on thyroid size and iodine content. The decreased thyroid iodine of rats fed this material and inadequate iodine appears to be due to a fall in the protein bound fraction. This may be caused by an increased thyroxine secretion rate. Further experiments are in progress further to purify and study the metabolism of the active principle.

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Immunochemical Determination of Orosomucoid,* (22121)

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A low molecular weight, acidic glycoprotein having the mobility of an alpha-1-globulin at pH 8.6 has been isolated from human serum and has been extensively characterized(1-5). This protein, designated orosomucoid(5,6), is a major component of the "seromucoid"(7) or "mucoprotein"(8) fractions of serum, fractions which increase significantly in a number of diseases(6).

Studies on the concentration of orosomucoid in serum and other body fluids, as well as in various tissues, may be expected to yield information relating orosomucoid to disease and bearing on the origin of this glycoprotein. An immunochemical determination seemed best suited to the determination of orosomucoid in solutions containing proteins having similar stability and solubility characteristics. Previous attempts to prepare precipitating antibodies to this protein have been only moderately successful(9,10), due to the low antigenicity of orosomucoid. In the present paper it is shown that precipitating antibodies against orosomucoid can be prepared in chickens, and that these can be used to quantitate orosomucoid in serum and in tissue extracts.

Materials and methods. Orosomucoid was prepared from fraction VI of pooled normal human serum[‡] by the method of Kefalides and Winzler(5). Antiserum was produced in healthy hens about one year old. One hundred mg of orosomucoid dissolved in 3 ml of water and mixed with an equal volume of

aluminum hydroxide adjuvant was injected into the breast muscle. Two weeks later the injection was repeated, and 7 days later the chickens were bled out by cardiac puncture. The blood was citrated to prevent coagulation and to obtain maximum yields, and the cells separated by centrifugation. One hundred fifty mg of solid sodium chloride was added for each ml of plasma. The suspension was centrifuged and the fibrinogen which was salted out was removed as a gelatinous mat at the surface. The serum was then diluted with an equal volume of water to make the final sodium chloride concentration about 8%. The procedures developed by Goodman *et al.*(11-13) were adapted for the present immunochemical studies. Qualitative tests were carried out by adding a constant amount of antiserum (0.2 ml of a 1:2 dilution of antiserum) to 0.2 ml portions of serial dilutions of antigen in 8% NaCl, and examining for the presence of a precipitate after 1 hour at 38°C and again after 18 hours at 4°C. Quantitative determinations were carried out in 8% NaCl by adding 0.5 ml of the antiserum preparation to 0.5 ml of the antigen dilutions. The reactants were incubated at 38°C for 1 hour and then placed in a refrigerator for 18 hours. The tubes were then centrifuged at 2500 rpm for 30 minutes. Supernatants were decanted and saved for tests for antigen or antibody excess. Precipitates were resuspended in 5 ml of 8% saline and centrifuged again. A control containing antigen and normal chicken serum, as well as one containing 8% saline and the antiserum were carried out with each series of determinations. In no case did significant precipitation occur in the controls. The amount of protein in the precipitates was determined either from nitrogen determination by the method of Lanni *et al.*(14), using a factor of 6.25 to convert nitrogen values to

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TABLE I. Quantitative Orosomucoid-Antiorosomucoid Interactions.

Orosomucoid, μg	Total protein in precipitate, μg	Orosomucoid precipitated, μg	Antibody precipitated (by difference), μg	AB/AG	Supernatant tests, excess
0	0	—	—	—	AB
.7	13.8	.7	13.1	19.2	"
1.4	26.6	1.4	25.2	18.0	"
2.8	50.0	2.8	47.2	16.9	"
5.5	75.0	5.5	69.5	12.6	"
11	100.6	11.0	89.6	8.2	"
21	126.2	—	—	—	AG
42	158.2	—	—	—	"
84	113.4	—	—	—	"
167	94.2	—	—	—	"

0.5 ml antiserum PC-7 (diluted 1:2) plus 0.5 ml orosomucoid solutions in 8% NaCl. (Incubation 1 hr, 38°C, 18 hr 4°C. Precipitated nitrogen determined and converted to protein by multiplying by 6.25.)

protein, or by the method of Lowry *et al.* (15) using serum albumin as the standard. The serum seromucoid fraction was determined by the method of Weimer and Moshin (16).

Results. The antigenicity of orosomucoid is rather low in comparison with other plasma proteins. Preparation of antiserum from different chickens varied markedly, containing from 100 to 400 μg of antibody protein per ml.[§] The preparation used to obtain the data shown in Table I contained between 90 and 100 μg of antiorosomucoid in 0.5 ml of the 1:2 dilution of the antiserum.

Table I shows a typical set of results when pure orosomucoid is mixed with antiserum prepared against it. As with other chicken precipitin systems (9) in which the reaction is carried out in concentrated NaCl, a relatively large excess of antigen is needed before inhibition of precipitation begins. The ratio of antibody to antigen in the precipitate formed in the antibody excess zone ranged from 19.2 to 8.2. Amounts of precipitate in this antibody excess zone, when plotted, serve as a calibration curve for determining the orosomucoid content of unknown mixtures. Since as little as 0.7 μg of the antigen yields a precipitate 19 times its own weight,

it is unlikely that the precipitate results from a reaction with a minor component of the antigen.

To ascertain the homogeneity of the orosomucoid-antiorosomucoid system, the precipitation reaction was studied in a gel medium by the technic developed by Oudin (17). Two types of antisera were employed in these studies, antiorosomucoid and antibodies against fraction VI from which orosomucoid was prepared. This fraction contains albumin as well as several α -1 and α -2 proteins in addition to orosomucoid. The results of the studies using the Oudin technic are summarized in Table II. Since single bands were observed in series 1, 2 and 3, it appears that the orosomucoid-antiorosomucoid system is a homogeneous one. This band had a rate of migration which was proportional to the square root of the time, as shown by Munoz and Becker (18).

TABLE II. Orosomucoid-Antiorosomucoid Interactions in a Gel System.

	In gel 1:2 dilution of anti- serum in 8% NaCl, 0.15 M pH 7.4 phos- phate & 0.6% agar	Overlying solution 1% sol. of antigen in 8% NaCl	Results
1. Antiorosomucoid	Orosomucoid		Single strong band
2. "	Fraction VI		<i>Idem</i>
3. Antifraction VI	Orosomucoid		Single weak band
4. "	Fraction VI		Four distinct bands

[§] It has been subsequently found that 4 to 8-fold higher antibody titers can be obtained by a single intramuscular injection of 200 mg of orosomucoid to chickens and collection of the blood in 10 days. It is likely that other injection schedules may result in still more effective antisera.

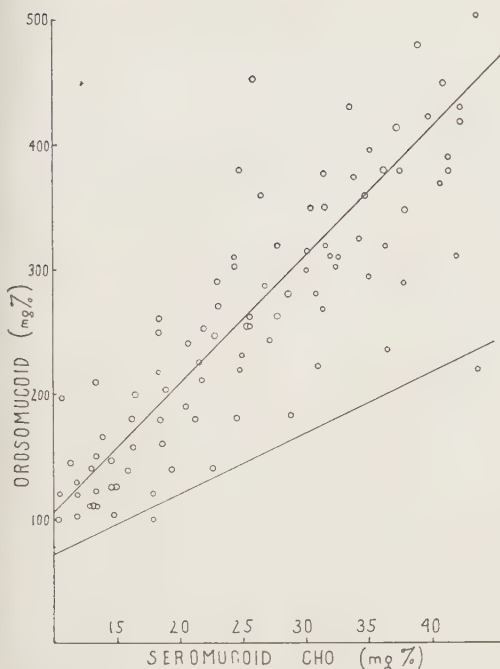


FIG. 1. Comparison of seromucoid fraction and orosomucoid levels in normal and abnormal sera.

The orosomucoid content of serum can be estimated by the immunochemical method described. Two-tenths ml of serum is diluted to 40 ml with 8% NaCl. Five-tenths ml of the diluted serum is added to 0.5 ml of 1:2 antiserum, and the incubation and protein determination carried out as previously described. The amount of orosomucoid is then determined from calibration curves of the type shown in Table I. Fifty normal sera examined in this manner showed a mean value and standard deviation of 103 ± 26 mg%. A single sample of serum was assayed repeatedly over the course of several months and gave consistent results. Thus, serum orosomucoid appears to be stable to storage by refrigeration.

In many clinical conditions including cancer, tuberculosis, etc., the orosomucoid levels are significantly elevated. Detailed data on serum orosomucoid levels in disease will be considered in subsequent reports. Values for orosomucoid are slightly higher than those for the seromucoid fraction determined in perchloric acid filtrates of serum(6). This is due primarily to the fact that there is con-

siderable (30-50%) coprecipitation of the seromucoid fraction with the proteins precipitated by perchloric acid. Since it has been shown that orosomucoid is a major component of the seromucoid fraction(19), studies on seromucoid and orosomucoid levels of a series of 100 normal individuals and patients with various diseases associated with high serum glycoprotein levels were carried out. The results, shown in Fig. 1, demonstrate a rough correlation between the serum levels of orosomucoid and of the seromucoid fraction. The lower line in Fig. 1 is that expected from orosomucoid values corrected to a carbohydrate basis by multiplying by .16 (the fraction of hexose in orosomucoid)(3). Since the values of the seromucoid fraction are low by 30 to 50% due to coprecipitation with other proteins during perchloric acid precipitation(8), a further correction of the seromucoid values has been made by multiplying by 0.6. This second correction, which depends in part upon the total protein value, results in the upper line shown in Fig. 1. The fact that most of the experimental points lie along this line suggests that orosomucoid is the major protein in the seromucoid fraction. This substantiates the observation to be reported elsewhere(19) in which the seromucoid fraction has been shown by immunological methods to contain orosomucoid.

Much of the deviation of individual points from the best straight line is undoubtedly due to differences in coprecipitation or to combined experimental errors of the two methods. Points far to the left of this line, however, may be due to a cross reaction of the antibody with proteins precipitated by perchloric acid.

The present results demonstrate that an increase in the levels of orosomucoid in the serum of patients with tuberculosis, cancer, and a variety of other diseases can be determined with a specific immunological method.

The specificity and ease with which this determination can be carried out should make it a useful adjunct for the study of the source and significance of the serum glycoproteins. A possible extension of this work involves the coupling of fluorescein isocyanate with the antibodies to orosomucoid and the investiga-

tion of the deposition of the labeled antibodies in freeze-dried sections of various tissues suspected of being sources of orosomucoid.

Summary. A homogeneous glycoprotein, orosomucoid, isolated from normal human serum was found to be weakly antigenic in chickens. The antibody-antigen system appeared to consist of a single antigen and its specific antibody. A procedure for determining immunochemically the orosomucoid content of serum has been described. The results show a reasonably good correlation of orosomucoid with the levels of the seromucoid fraction (mucoprotein) of normal and pathological serum.

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Inhibition Studies with *Streptococcus faecalis*. Quinolines. (22122)

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Farber and his associates(1) observed that the anti-folics may be used to treat leukemia. This observation gave impetus to the synthesis and examination of metabolite blocking compounds. One outgrowth of this was the observation by Hitchings and his co-workers (2,3) that some anti-folics may have anti-malarial properties. In view of these findings we have examined compounds initially prepared for testing against malaria(4-9) for their ability to interfere with the utilization of folic acid.

In this communication the results of tests on 93 quinolines for anti-folic properties are given. It may be indicated at the outset that some of the most useful anti-malarials are without effect against folic acid. While some

specificity of activity was suggested by the study, the compounds do not compare favorably with some of the known anti-folics. Accordingly, the results appear to be only of theoretical interest at this time.

Experimental. The method of test was based on the use of *Streptococcus faecalis* (ATCC No. 8043) with a slightly modified version of the medium of Teply and Elvehjem (10). The modifications consisted of omitting the xanthine from the medium and of substituting "vitamin-free" casein hydrolysate (Nutritional Biochemicals) for the charcoal treated preparation suggested by the above authors. Under our conditions of test, with increasing amounts of folic acid in the medium up to 1 μg per ml of medium, the

TABLE I. Quinolines Which Interfere with the Utilization of Folic Acid.

Compound*	Derivatives	Activity vs. folic acid, $\mu\text{g/ml}$
4-NH(CH ₂) ₂ N(C ₆ H ₁₃) ₂	3-CH ₃ 7-Cl(4)	1.6/ 8
4-NH(CH ₂) ₄ N(C ₂ H ₅) ₂	3-NO ₂ 7-Cl	40 /200
4-CH(C ₆ H ₅)COO(CH ₂) ₂ N(C ₂ H ₅) ₂ (8)		"
4-COO(CH ₂) ₂ S(CH ₂) ₂ N(C ₂ H ₅) ₂	2-OC ₄ H ₉	"
4-COO(CH ₂) ₂ S(CH ₂) ₈ N(C ₂ H ₅) ₂	"	"
4-SCH ₂ CONH(CH ₂) ₂ N(C ₂ H ₅) ₂	3-CH ₃ 7-Cl(9), 3-Br 7-Cl(9), 3-CH ₃ 6-OCH ₃ (9), 3-CH ₃ 6-Br(9)	"
8-NH(CH ₂) ₃ N(C ₂ H ₅) ₂	6-OCH ₃	8 / 40

* Six publications, considered representative, are cited for the chemistry of compounds given in the Tables. A more complete listing of compounds tested against malaria may be seen in Wiselogle(12) or in the F. S. A. monograph(13).

growth, as judged by acid production, increased from blanks of about 0.5 ml of N/10 alkali per tube up to 9.5 to 10 ml of alkali. For the test, about half maximum growth was obtained with 0.32 μg of folic acid per ml of medium. Accordingly, the test compounds were run against this amount of folic acid in the medium. They were added to duplicate tubes in amounts of 1.6, 8.0, 40 or 200 μg per ml of medium. The tubes were plugged, sterilized for about 12 minutes at 120°C, cooled, inoculated(11) with a drop of a suspension of the organism, and incubated for 3 days at 37°C. At the end of this time, the growth was estimated titrimetrically.

Results. The compounds tested and their ability to interfere with the utilization of folic acid are summarized in Tables I and II. An arbitrary ratio term is given in the Table to evaluate the compounds which interfere with the growth of the organism. The lesser amount is that at which little or no interference with the growth of *S. faecalis* occurred, while the larger amount is that at which growth was essentially completely prevented.

From the results summarized in Table I it may be seen that 10 of the quinolines were toxic to the organism. To determine the nature of the toxicity, the inhibitory activity was further differentiated. With 2.0 μg of citrovorum factor(14) per ml of medium as the limiting nutrient, in place of folic acid, the mercapto quinoline N-(2 diethylaminoethyl) - (7-chloro-3-methylquinolyl-4) - mercaptoacetamide was no longer inhibitory. With 2.5 μg of thymine(15,16) per ml of medium as the limiting nutrient in place of

folic acid 7 - chloro-4-(4-diethylaminobutyl-amino)-3-nitroquinoline was no longer inhibitory. Finally, with 2.5 μg of thymidine(17) per ml of medium as the limiting nutrient in place of folic acid, the mercaptoquinoline N-(2 - diethylaminoethyl) - (6 - methoxy-3-methylquinolyl - 4) - mercaptoacetamide was no longer inhibitory.

The inhibitory properties of the foregoing 3 quinolines, while interesting, are considerably below those of some of the better known anti-folics. For example, in the presence of 0.32 μg of folic acid per ml of medium under the above conditions of test the inhibition ratios were 0.008/0.04 μg for aminopterin, 0.00032/0.0016 μg for amethopterin, and 0.0016/0.008 μg for pyrimethamine (Daraprim) per ml of medium. Thus the latter were 5000-fold (or more) as active as the above 3 quinolines. Accordingly, the results appear to be of no more than theoretical interest at this time.

Seven of the quinolines of Table I which blocked the utilization of folic acid were still inhibitory with thymidine in the medium as the limiting nutrient. Whether the inhibitory activity of these compounds was due to a block in the nucleic acid metabolic sequence or whether it was due to interference in some other metabolic sequence is not known.

The anti-malarials given in Table II, in amounts up to 200 μg per ml of medium, did not inhibit the organism in the presence of limiting amounts of folic acid. This included chloroquine (Aralen), hydroxychloroquine†

† Not to be confused with oxychloroquine.

(Plaquenil), sontoquine, primaquine, and isopentaquine. The biguanide anti-malarial Paludrine in our hands was likewise inert, although it is clearly an anti-folic with the *L. casei* test system(18). Thus, the foregoing

anti-malarials differ from pyrimethamine, which is a strong inhibitor of folic acid with *S. faecalis*.

Accordingly, it may be concluded that an examination of 93 quinolines, prepared in

TABLE II. Quinolines Which Do Not Interfere with the Utilization of Folic Acid.

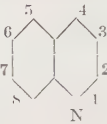
Compound		Derivatives
a. 4-amino quinolines		
bis [(4-NH ₂ ,2-CH ₃ ,6-quinoly1)-NHCO] = C 		
4-NH(CH ₂) ₂ NHCH ₂ CH ₂ OH		7-Cl, 3-CH ₃ , 7-Cl
" N(C ₂ H ₅) ₂		3-CH ₃ 7-Cl
" N(C ₄ H ₉) ₂		"
4-NH(CH ₂) ₃ N(C ₂ H ₅) ₂		7-Cl(5), 3-CH ₃ 7-Cl (brachysan)
4-NHCH ₂ CHOH CH ₂ N(C ₂ H ₅) ₂		7-Cl (oxychloroquin) (6), 3-CH ₃
		7-Cl(6), 3-Br 7-Cl, 3-I 7-Cl, 5-Cl
4-NHCH(CH ₃)(CH ₂) ₂ NC ₄ H ₉ O		7-Cl
4-NH(CH ₂) ₄ N(C ₂ H ₅) ₂		7-Cl(5), 7-Br(5), 6-OCH ₃ , 3-CH ₃
		7-Cl (norsontoquine) (6)
4-NH(CH ₂) ₄ N(C ₄ H ₉) ₂		3-CH ₃ 7-Cl
" 2CHOHCH ₂ N(C ₂ H ₅) ₂		7-Cl
4-NHCH(CH ₃)(CH ₂) ₃ N(C ₂ H ₅) ₂		" (chloroquine) (5), 7-Br(5),
		3-CH ₃ 7-Cl (sontoquine) (6), 3-CH ₃
" N(C ₂ H ₅)(C ₂ H ₄ OH)		7-Br(6), 2-CH ₃ 7-Cl, 5-Cl
" NC ₄ H ₉		7-Cl (hydroxychloroquine)
4-NHCH ₂ CH(C ₆ H ₄ OCH ₃)(CH ₂) ₂ N(C ₂ H ₅) ₂		7-Cl
" (C ₆ H ₅)(CH ₂) ₂ N(C ₂ H ₅) ₂		3-CH ₃ 7-Cl
4-NH(CH ₂) ₂ S(CH ₂) ₂ N(C ₂ H ₅) ₂		"
" SO ₂ (CH ₂) ₂ N(C ₂ H ₅) ₂		7-Cl(7)
b. Carbon linked at position 4		
4-CH(C ₆ H ₅)CONH(CH ₂) ₂ N(C ₂ H ₅) ₂		7-Cl(8)
" (CH ₂) ₂ N(CH ₃) ₂		5-Cl(8)
" (CH ₂) ₂ N(C ₂ H ₅) ₂		7-Cl(8)
4-C(C ₆ H ₅)(CN)(CH ₂) ₂ N(CH ₃) ₂ (8)		" , 6-OCH ₃ (8)
" (CONH ₂)(CH ₂) ₂ N(CH ₃) ₂		5-Cl(8)
" (CONH ₂)(CH ₂) ₂ N(C ₂ H ₅) ₂		7-Cl(8)
4-CH(C ₆ H ₅)COO(CH ₂) ₂ N(C ₂ H ₅) ₂		"
c. 4-mercaptoquinolines		
4-SCH ₂ CONH(CH ₂) ₂ N(C ₂ H ₅) ₂ (9)		7-Cl(9), 2-CH ₃ 7-Cl(9), 5-Cl(9),
		3-CH ₃ (9), 3,8-(CH ₃) ₂ (9), 3-CH ₃
4-S(CH ₂) ₃ N(C ₂ H ₅) ₂		8-OC ₂ H ₅ (9), 6-OCH ₃ (9)
" NC ₅ H ₁₀		7-Cl(4), 3-CH ₃ 7-Cl(4)
4-SCH ₂ COO(CH ₂) ₂ N(CH ₃) ₂		7-Cl(4)
" N(C ₂ H ₅) ₂ (9)		7-Cl(9), 5-Cl(9)
" NC ₅ H ₁₀		" , 6-OCH ₃ (9)
" NC ₄ H ₉ O		5-Cl(9)
4-SCH(CH ₃)COO(CH ₂) ₂ N(C ₂ H ₅) ₂		7-Cl(9)
4-S(CH ₂) ₅ NHC ₃ H ₇		" (4)
4-S(CH ₂)COO(CH ₂) ₂ N(C ₂ H ₅) ₂		" (9)
4-S(CH ₂) ₂ COO(CH ₂) ₃ N(C ₂ H ₅) ₂		" (9)
d. Oxygen linked at position 4		
4-OH		3-CH ₃ (6), 7-Cl 2-COOH(5)
4-OCH(CH ₃)(CH ₂) ₃ N(C ₂ H ₅) ₂		7-Cl(4)
4-O(CH ₂) ₂ S(CH ₂) ₂ N(C ₂ H ₅) ₂		7-Cl

TABLE II (continued).

Compound	Derivatives
e. 8-amino quinolines	
8-NH ₂	6-OCH ₃ (amichin)
8-NHCH(CH ₃)CH ₂ NHC ₃ H ₇	6-OCH ₃
8-NH(CH ₂) ₃ N(C ₂ H ₅) ₂	"
" (CH ₂) ₁ NH ₂	"
8-NHCH(CH ₃)(CH ₂) ₃ NH ₂	" (primaquine), 4-CH ₃ 6-OCH ₃
" NHC ₃ H ₇	" (isopentaquine)
" N(C ₂ H ₅) ₂	6-OH
8-NH(CH ₂) ₅ NH ₂	6-OCH ₃
" (CH ₂) ₂ S(CH ₂) ₂ N(C ₂ H ₅) ₂	" (7)
" (CH ₂) ₃ S(CH ₂) ₂ N(C ₂ H ₅) ₂	" (7)
" S(CH ₂) ₃ N(C ₂ H ₅) ₂	"
f. 2-mercapto quinolines	
2-S(CH ₂) ₂ N(C ₂ H ₅) ₂ (4)	
" (CH ₂) ₃ N(C ₂ H ₅) ₂	4-CH ₃ (4)
g. Carbon linked at position 1	
1-CH ₂ C ₆ H ₄ SO ₂ NH ₂	1-Br

connection with an anti-malarial program, has not disclosed compounds with immediate application.

Summary. 1. Ninety-three quinolines were tested against *Streptococcus faecalis* in the presence of limiting amounts of folic acid. Ten of the quinolines were toxic to the organism when they were in the medium at concentrations of 8.0 to 200 µg/ml. The toxicity of 3 of the 10 inhibitors could be abolished. The inhibition of N-(2-diethylaminoethyl)-(7-chloro-3-methylquinolyl-4) - mercaptoacetamide was reversed by citrovorum factor, that of 7-chloro-4-(4-diethylaminobutylamino)-3-nitroquinoline was abolished by thymine, and that of N-2-diethylaminoethyl-6-methoxy-3-methylquinolyl-4) - mercaptoacetamide was overcome by thymidine. The remaining 7 were not studied further since their degree of inhibition did not compare sufficiently favorably with that of some of the better known anti-folics. 2. None of 5 anti-malarials examined (chloroquine, hydroxychloroquine, sontoquine, primaquine, and isopentaquine) interfered with the utilization of folic acid. Thus, these differ metabolically from pyrimethamine which has been shown to be both an anti-folic and an anti-malarial.

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Development of Spontaneous Hepatoma in Mice in Relation to Composition of Dietary Fat.*† (22123)

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The development of spontaneous hepatomas in C₃H mice has been shown to be enhanced by an increased amount of fat in the diet(7,10). Very little, however, is known about the dependence of this tumor on the nature of the dietary fat, of the type demonstrated for the azodye induced hepatoma of the rat(8). In the present study an attempt has been made to find if groups of fat components, derived from the same source, but separated from each other by physical means and fed in equivalent amounts would exert a specific action on the development of the mouse hepatoma.

Methods. Among several methods of fat fractionation(1-5) fractional crystallization from solvents(2,4) presents decisive advantages for nutritional studies. It does not subject fat to chemical reactions, the exact outcome of which it is impossible to predict without precise knowledge of composition of the original fat. It does not involve addition of more than one chemical, which if properly chosen as to its boiling point can be quantitatively removed. Nor does it require heating the fat to higher temperatures, as *e.g.*, molecular distillation. The procedure adopted was as follows: One pound of hydrogenated vegetable fat (H.V.F.)† was melted at 50°C, then dissolved in 2 lb of diethyl ether (Purified ether reagent, Merck, glass containers). Ether was chosen as solvent, rather than acetone, because of its lower boiling point. The resulting mixture had a temperature of 35°C. The mixture was cooled for 30-40 minutes under constant mechanical stirring until a temperature of +15°C was reached. The precipitate formed was filtered by gravity at that temperature (Fraction I), filtration re-

quiring about 20 minutes. E. & D. filter paper No. 615, size 33 was used. Precipitate was not washed, but the mother liquor was pressed out as far as possible. The filtrate was cooled further and precipitate formed at +5 (Fraction II), -5 (Fraction III) and -15° (Fraction IV) filtered off similarly. Cooling times required were approximately 40, 50 and 180 minutes, respectively. Filtration time was approximately the same for all fractions. The precipitate obtained at the last temperature constituted "Fraction V." The residues were heated at low pressure until liquefied and remaining ether removed by vacuum distillation at the temperature of liquefaction. This required approximately 4 hours. The filtrate at -15°C was similarly freed of ether at 35°-40°C. Crude fractions thus obtained were allowed to solidify and kept refrigerated until their incorporation into diets. Titer ranges were determined on aliquots of each fraction and are given in Table I. Total yields for each fraction were determined on each lot processed and were found to vary little from one processing to another. Average values are also given in Table I. No attempt has been made to determine exact composition of individual fractions. However, on the basis of extensive analyses of fractions obtained by similar methods reported by other workers it is believed that fractions precipitating at higher temperatures contained a higher proportion of glycerides of long-chain, branched-chain

TABLE I. Melting Ranges and Approximate Recoveries of Hydrogenated Vegetable Fat Fractions.

Fraction	Melting range (°C)	Recovery* (g)
I	55-57	1380
II	44-49	1060
III	37-41	1120
IV	30-33	1640
V	12-26	2000

* Abstract in *Proc. of Am. Assn. of Cancer Research*, 1954, v1, 22.

† This work has been supported in part by grant from Minn. Division of the Amer. Cancer Soc.

‡ H.V.F. = commercial Crisco.

* Initial amount of material approx. 15000 g.

TABLE II. Incidence of Hepatomas.

	H.V.F.	Lard	Fr. I	Fr. II	Fr. III	Fr. IV	Fr. V	P*
No. of tumor bearing mice	8	9	3	0	5	9	13	<.001
No. of mice bearing multiple tumors	1	3	1	0	1	1	3	
Total No. of tumors	9	12	4	0	6	12	20	
Size: <10 mm	4	6	0	0	2	8	12	
>10 mm	5	6	4	0	4	4	8	

* By the χ^2 method.

H.V.F. = Hydrogenated vegetable fat.

Fr. = Fraction.

and saturated fatty acids and a smaller proportion of unsaponifiable matter. Composition of diet into which fractions were incorporated was as follows: casein 29%, glucose 34%, yeast 8.2%, Jones Foster salt mixture 4.1%, alfalfa leaf 4.1%, cod liver oil 2.1%, wheat germ oil 1%, neutral fat (lard, hydrogenated vegetable fat or one of the 5 fractions) 17.5%. The diet was made in batches sufficient to last for 3 months at a time. Fractionation was carried out each time a new batch of diet was required. Seven groups of 20 male C₃H mice each were used. Five were fed fractions of hydrogenated vegetable fat while 2 control groups received unfractionated and untreated H.V.F. and lard, respectively. Animals were started on experimental diets at weaning (3 weeks of age, average weight about 10 g). Members of the

same litter were distributed among as many groups as possible. Animals were kept 2 to a cage and weighed at weekly intervals. They were fed *ad libitum* from a common food cup, which was left in the cage for one week at a time. At all times they had access to water. All cages were placed in the same part of the room and room temperature did not vary more than 8°F from day to day.

Results. The experiment was terminated after 54 weeks and autopsies performed on all animals. Table II gives the cumulative incidence of hepatomas up to that time. It also summarizes the occurrence of multiple tumors and the breakdown according to size. It can be seen that fractions of low melting point show a markedly greater incidence of hepatomas. Compared with fractions of high melting point there appears to be little corre-

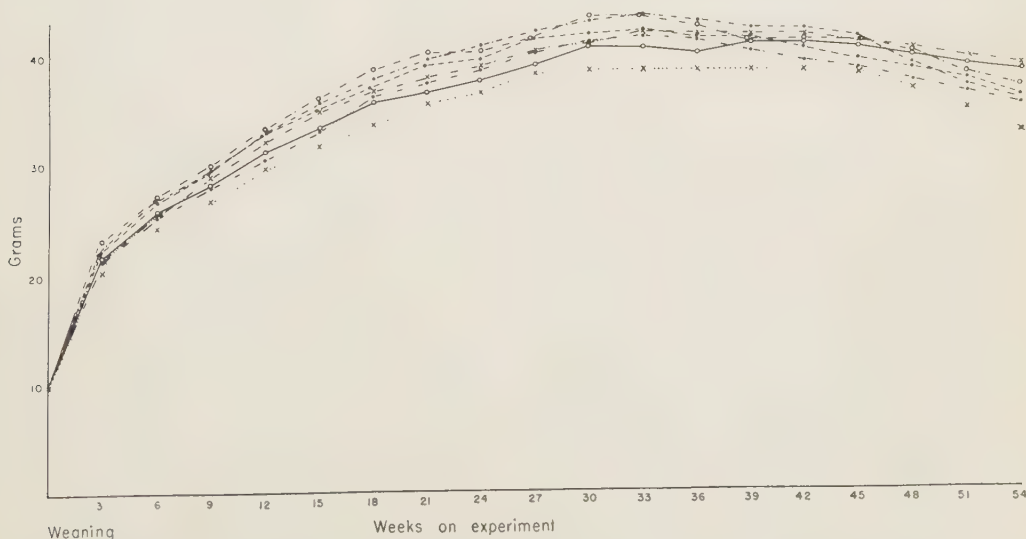


FIG. 1. Weight curves of mice fed different fractions of hydrogenated vegetable fat (H.V.F.). — lard, —..... H.V.F., ... Fraction I, —...— Fraction II, —.— Fraction III, ——— Fraction IV, —..— Fraction V.

lation between tumor incidence and tumor size. The occurrence of multiple tumors is sufficiently rare in all groups so as not to permit any conclusions as to the presence of differences.

The foregoing data strongly suggest that the nature of the dietary fat may have a significant bearing on the development of the C₃H mouse hepatoma. The effect observed here is similar to the one reported by Shear and Leiter(9), who fractionated lard by filtration at 38°C and found that the liquid fraction when used as a carrier for benzpyrene gave rise to more tumors than the solid fraction.

It still remains to be established however, whether the differences in the effects are due to qualitative peculiarities of the different fractions or to differences in the actual amounts of fat absorbed. No simple correlation between weight and tumor incidence is apparent from a comparison of the weight curves given in Fig. 1 and the data in Table II. This would suggest a qualitative, rather than a quantitative, difference, but the question as to the nature of the responsible factor remains open for the present.

Summary. Five fractions of hydrogenated vegetable fat (commercial crisco) have been prepared by crystallization from ether at +15, +5, -5, and -15°C. When incorporated in the standard diet at equivalent

levels, fractions of low melting point, presumably containing largely unsaturated and shorter-chain fatty acids as well as unsaponifiable matter, are found to give rise to a high proportion of hepatomas in C₃H male mice, while fractions of high melting point give rise to but a few. The magnitude of the observed difference in incidence ($p < .001$) is believed sufficient to justify further studies on the nature of the responsible factors.

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Collagenase Activity of Dog Pancreatic Juice. (22124)

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Pancreatic juice obtained from pancreatic fistulae artificially created in dogs produces striking digestion of collagen. This is not due to trypsin, chymotrypsin, or carboxypeptidase. It is the purpose of the present work to describe these studies.

Methods and materials. Adult mongrel dogs of both sexes were used. Under aseptic technic the abdomen of the anesthetized animal was opened through a midline incision

and the pancreas exposed. The accessory pancreatic duct was dissected free, ligated, and cut, following which the main duct was identified. After placing a ligature close to the point of its entrance into the duodenum, the duct was cannulated with a polyethylene tube whose lumen measured 1.14 mm in diameter; this was tied securely in place. The tube was then brought out through a separate stab incision, following which a Witzel jejun-

ostomy was performed using a number 8 French rubber catheter which was brought through another stab incision. The wound was then closed in layers and a sterile condom was tied to the polyethylene tube. The jejunostomy tube was then securely clamped and a dressing applied. Following recovery from the anesthetic, secretions started flowing in 12-24 hours from the polyethylene tube reaching a maximum of 20 ml per hour in 36-48 hours. The animals could be kept alive for prolonged periods if the pancreatic secretions were returned through the jejunostomy tube. Otherwise they became dehydrated, anorexic and lethargic and died within 7 to 8 days. The secretions tested were collected within the first 24-48 hours in each instance and were also tested for sterility by routine bacteriologic methods. These were free of bacterial growth. Collagen was prepared by the method of Buechler(1) using fresh beef achilles tendon instead of steer hide. 100 mg of prepared tendon was suspended in 2.0 ml of secretion from a pancreatic fistula and incubated at 37°C for 5 hours. The suspension was then filtered and the precipitate washed. The precipitate was then dissolved in 9.0 ml of 0.5 N HCl and autoclaved for 30 minutes at 15 lb pressure. The solution was then made up to 10.0 ml volume. Collagenase activity was measured by the quantitative assay of the hydroxyproline in the residue as described by Neuman and Logan(2). Since hydroxyproline forms a constant fraction of collagen, this method is an accurate assessment of the amount of undigested collagen, permitting ready calculation of the amount of collagen digested. Commercially available preparations* of trypsin, chymotrypsin, and carboxypeptidase were used. 0.1 M phosphate buffers at pH 7.0 and 8.0 were used. 0.5 mg of each of the commercial enzymes were dissolved in 2.0 ml of buffer solution for testing the activity. Changes in collagen apparently occur at pH

TABLE I. Percent Digestion of Collagen and Casein by Pancreatic Juice and Trypsin.

		% collagen digested	% casein digested
Pancreatic juice	Dog 1	52	0
	2	41.5	0
	3	45	0
Trypsin before heating	No. 1	16	96
	2	13	92
Trypsin after heating	No. 1	0	85
	2	0	79

4.5 and below and will permit digestion by trypsin and vegetable cathepsins(3). These findings have been confirmed by us and are not included in this report.

Results. Unbuffered pancreatic juice was used in the tests on collagen. The usual pH range was 8.2-8.6. The digestion of collagen by dog pancreatic juice varied. Dog No. 1 digested 52.0%, No. 2 digested 41.5%, and dog No. 3, 45.0%. (Table I). Commercially prepared trypsin, both at pH 7 and 8 had slight collagen digesting ability. Preparation No. 1 digested 16.0% and preparation No. 2 digested 13.0%. As pointed out by Northrop(4) and confirmed by us, heating to 95°C for 4½ minutes, then cooling to 20°C has little effect on the proteolytic activity of trypsin, as determined by the method of Grob(5). However, such heating markedly alters its ability to digest collagen. Neither trypsin preparation digested any collagen after such heating (Table I). When pancreatic juice was heated in the same fashion all collagenase activity disappeared. The inactivated pancreatic juice had no tryptic activity. Bacterial collagenase prepared from *Cl. histolyticum* was heated to 95°C for 4½ minutes then cooled, with complete loss of collagenase activity. Chymotrypsin and carboxypeptidase did not digest collagen at pH 7.0 or 8.0. Duodenal content, extract of duodenal mucosa and bile had little effect on the collagenase activity of the pancreatic secretion. The activating substance, if any, for collagenase activity remains unidentified.

Discussion. The marked collagenase activity of crude pancreatic juice was present in each of the dogs tested. This cannot be accounted for by the action of any of the pre-

* Commercial preparations used: Trypsin preparation no. 1: Tryptar, Armour Lab.; trypsin preparation no. 2: Trypsin, Worthington Biochemical Co., 2x crystallized Chymotrypsin, Worthington Biochemical Co.; Carboxypeptidase, 3x crystallized.

vously described proteolytic enzymes of the pancreas for 3 reasons: 1. The degree of digestion is at least 3 times as great as that obtained from trypsin. 2. Heating trypsin to destroy its collagen digesting ability, but not its proteolytic properties, reduces the amount of collagen digested to zero. 3. Pancreatic juice from a dog fistula which has not been activated by intestinal juice has no tryptic activity, nevertheless, it has marked collagenase activity. The inability of trypsin to digest any significant amount of collagen has also been reported by Neuman and Tytell(6).

These findings suggest that collagenase must be considered in the pathogenesis of pancreatitis and other pancreatic diseases.

Summary. Evidence has been presented to

show that pancreatic juice of the dog has the ability to digest collagen. This ability is not due to the proteolytic enzymes of the pancreas, trypsin, chymotrypsin, or carboxypeptidase.

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Effects of Thyroid Hormone and Propylthiouracil on Eruption Rate of Upper Incisor Teeth in Rats. (22125)

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Though the rat's incisor teeth continue to erupt throughout life, they are kept to a fairly constant length by the process of attrition. The rate of eruption, that is, the linear quantity by which the incisor teeth extrude into the oral cavity in a unit of time, is subject to alteration by hormonal action. Hoskins(1) found that thyroid hormone caused the incisors of newborn rats to erupt earlier than those in untreated control rats. This observation was confirmed by Karnofsky and Cronkite(2). Herzberg(3) noted that thyroid hormone caused an increase in the incisor eruption rate (I.E.R.) in adult rats; however, he offered no data to substantiate his claim. Baume, Becks and Evans(4) reported that ablation of the thyroid in adult rats produced a decrease in the I.E.R. In conformity with this view it seemed of interest therefore to extend these data by 1) quantitating the effect of thyroid hormone on the I.E.R. of adult rats and 2) testing the effect of a thyroid blocking agent, propylthi-

ouracil (PTU) on I.E.R. The latter procedure has the advantage that it leaves the parathyroid glands in a functional state. In many of the earlier studies the parathyroids were removed indiscriminately at the time of surgical thyroidectomy.

Procedures. Twenty-three male white rats (Holtzman strain), 80 days of age, were treated as follows: 8 animals were fed ground rat chow containing 0.5% desiccated thyroid; 8 rats were fed processed chow containing 0.15% PTU and 7 animals, which served as untreated controls, were fed only ground rat chow. Four rats on PTU-feeding were shifted at the end of the fifth week to a diet of ground rat chow containing instead 0.5% desiccated thyroid for an additional 2 weeks. The I.E.R. of all animals was measured according to the method of Schour(5) once each week during the course of the experimental period. Dental roentgenograms of upper incisors of rats fed thyroid were taken after 2 weeks on the diet, and those of the animals receiving PTU were taken after 5

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TABLE I. Effect of Desiccated Thyroid and Propylthiouracil on Incisor Eruption Rate of the Rat.

Group	Wk I			Wk II			Wk III			Wk IV			Wk V		
	No.	I.E.R., mm/wk	Wt, g	No.	I.E.R., mm/wk	Wt, g	No.	I.E.R., mm/wk	Wt, g	No.	I.E.R., mm/wk	Wt, g	No.	I.E.R., mm/wk	Wt, g
Control	7	2.6 ± .15*	256	7	2.7 ± .33	268	7	2.6 ± .20	260	7	2.3 ± .24	273	7	2.3 ± .22	270
Thyroid, .5%	8	3.1 ± .24†	232	8	3.5 ± .24†	220	7	3.0 ± .37	193	6	3.1 ± .40	197	5	2.6 ± .36	190
PTU, .15%	8	2.4 ± .14	233	8	1.95 ± .35	237	8	1.5 ± .20	240	8	1.2 ± .39	225	8	1.2 ± .28	219

* Stand. dev.

† T test performed for difference of I.E.R. of thyroid group from control group for Weeks I and II; $P = < 0.01$ —highly significant.

weeks of treatment. These were compared with corresponding films of the controls at the respective times.

Results. Feeding of desiccated thyroid (0.5%) admixed in ground chow caused a significant increase ($P = < .01$) in the I.E.R. after one week, and this rose to a maximum at 2 weeks when the I.E.R. was 30% above that manifested by the control animals (Table I). Thereafter the I.E.R. declined gradually despite continued thyroid feeding and by the fifth week had returned almost to the level of that of the controls. These thyroid-fed animals manifested a severe weight loss, most of which occurred during the period of the second through the third week of treatment. From the second through the fifth weeks of the experiment 3 rats of this group died. There were no deaths in the other experimental groups during this period.

The inclusion of 0.15% PTU in the diet produced a slowing of the I.E.R. which was significant by the second week and which continued to decrease until the fourth week when it reached a minimum of 48% of that observed in the control group. The I.E.R. did not decrease further even though PTU feeding was continued through the fifth week (Table I).

When desiccated thyroid 0.5% was substituted in the diet of 4 of the above animals which had received PTU for the previous 5 weeks, the I.E.R. increased within one week to 260% over that which these same animals had registered the previous week (Table II). Thyroid feeding was continued for an additional week and the increase in I.E.R. was maintained.

The upper incisors of the animals treated with PTU for 5 weeks possessed an increased amount of dentine per unit area of incisor. (Fig. 1). This had occurred at the expense of

TABLE II. Effect of Thyroid Hormone on I.E.R. of 4 Rats Previously Treated with PTU.

Treatment	I.E.R.	
PTU, 0.15% in diet, wk I-V	mm during wk V,	1.0 ± .24
Thyroid, 0.5% in diet, wk VI & VII		VI, 3.6 ± .18 VII, 3.5 ± .20

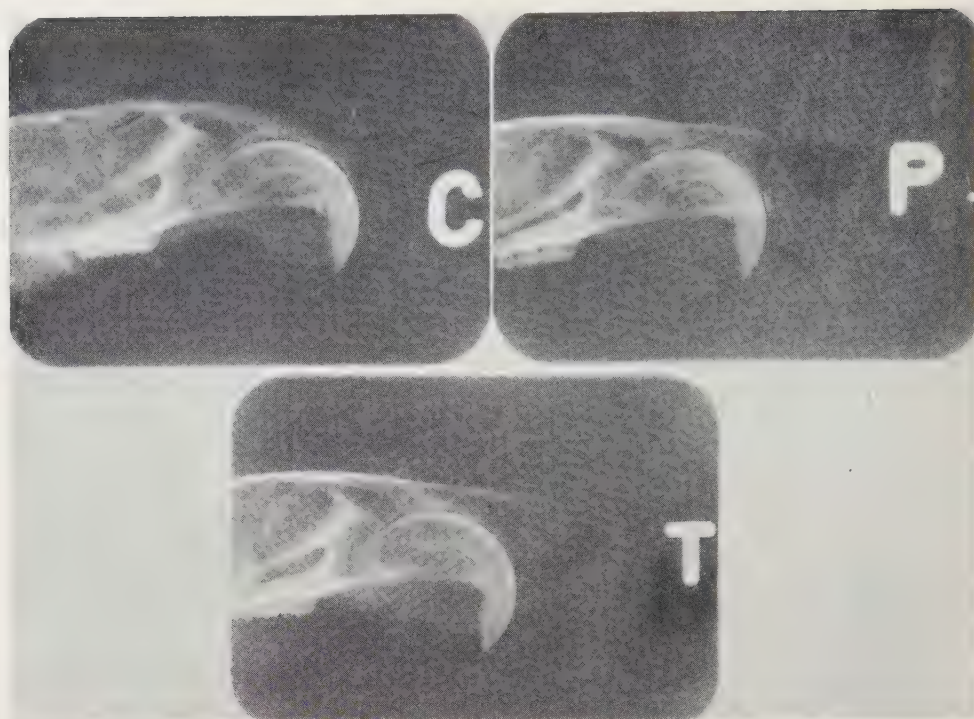


FIG. 1. Roentgenographs of incisors: C—Control animal. P—After 5 wk propylthiouracil feeding showing an increased amount of dentine which partially obliterates pulp chamber. T—Two wk of thyroid feeding showing enlarged pulp chamber associated with decreased amount of dentine per unit area of tooth.

the pulp chamber which was partially obliterated. Conversely the incisors of the thyroid-treated rats had large pulp chambers with a decreased amount of dentine by comparison with the films of control animals. It is to be noted that the roentgenograms were taken at the time when the maximum effects of the experimental treatment on the I.E.R. were demonstrated, *i.e.*, the second week of desiccated thyroid- and the fifth week of PTU-feedings.

Discussion. These data confirm the statement of Hertzberg that thyroid hormone increases the I.E.R. in normal rats. The response was most marked during the first two weeks of treatment and thereafter subsiding toward normal. An explanation of this phenomenon may be that in the first 2 weeks the effects on eruption are those of increased thyroid hormone action in healthy animals, whereas with continued thyroid treatment the toxic effects of the increased dosage, manifested by severe weight loss as well as the death of some of the treated animals, are

noted. That the effects of thyroid hormone administration are accumulative is well known. Although the eruption rate decreases from the peak reached in the first two weeks of treatment it does not return to the rate measured in the control animals.

The increased size of the pulp chambers of the incisors of the thyroid-fed group is probably due to the increase in the I.E.R. with no apparent change in the rate of dentinogenesis. There is therefore less time for dentine to be deposited per unit area of tooth with the result that there is less encroachment upon the pulp chamber. Conversely the roentgenograms of the upper incisors of animals given PTU demonstrate an obliteration of a large portion of the pulp chamber by dentine. A likely explanation here is that while the I.E.R. is decreased the rate of dentinogenesis is apparently not affected, hence the period during which dentine can be laid down per unit area of tooth is extended. This is also in agreement with the histological evidence

from the thyroidectomized animals of Baume *et al.*(4).

The increased sensitivity of the eruptive mechanism to thyroid hormone manifested by the PTU rats is probably due to the hypothyroid state of these animals and thus the end organs which are normally affected by this hormone become more reactive.

PTU causes a chemical thyroidectomy and produces a retardation of the I.E.R. which confirms the work of Baume *et al.*(4) in demonstrating a decreased eruption rate in the thyroidectomized adult rat. Since PTU does not affect the parathyroid gland, it is evident that the reduced I.E.R. can only be due to the lack of thyroid hormone.

Conclusions. Thyroid hormone is essen-

tial for a normal rate of incisor eruption. An excess of this hormone will cause an increased eruption rate. The use of the incisor eruption rate as a tool for demonstrating hormonal activity should be considered.

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Relative Pathogenicity of Newly Isolated Strains of Poliomyelitis for Monkey Kidney Cells and Mice. (22126)

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Adaptation of poliomyelitis viruses to passage in mice has been accomplished only with difficulty or after laboratory manipulation of newly isolated strains(1-7). Quantitative comparisons of the pathogenicity of poliomyelitis viruses in tissue culture and in animals have usually been carried out with well adapted laboratory strains(8-11) or have been limited because of the necessary use of large numbers of monkeys(12).

The purpose of this study is to compare the quantitative relationships of the ability of freshly isolated polio virus strains to cause cytopathogenic effect in monkey kidney epithelium in tissue culture and paralysis in mice. For this purpose a number of strains of each of the three types of polio virus isolated in tissue culture from stools of patients during a national survey in 1952 was available for this study(13).

Materials and methods. Ten percent suspensions of stools were prepared in a mortar

with phosphate buffered saline containing 2500 units of penicillin and 1250 μ g of streptomycin per ml, clarified by centrifugation at 8,000 r.p.m. for one-half hour, and the supernates used as inocula. Primary isolations were made in cultures of monkey kidney, either in flasks of suspended tissue fragments, according to the method of Weller, Enders, Robbins and Stoddard(14), with subsequent passage in roller tubes, or directly in monolayer roller tubes, prepared by the method of Youngner(15). Isolates were typed in roller tubes by neutralization with specific monkey antisera. All inoculations of mice and titrations in both mice and kidney roller tubes were carried out on virus strains in the first or second tissue culture passage from the original stool. Groups of ten 4-5-week-old CFW mice were inoculated intraspinally (I.S.) with 0.02 ml, and observed for 21 days. Type 2 isolates were also inoculated intracerebrally (I.C.), in a dose of 0.03 ml, into 10 mice each, which were observed for 28 days. Wherever indi-

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TABLE I. Results of Mouse Inoculation of Polio Strains in 1st or 2nd Monkey Kidney T.C. Passage.

Type	Intraspinal			Intracerebral		
	No. tested	No. pos.	% pos.	No. tested	No. pos.	% pos.
1	51	0	0	—	—	—
2	56	45	80	56	11	20
Original stools	39	6	15	6*	1	17
3	20	0	0	—	—	—

* All 6 were positive by I.S. inoculation.

cated, tissue culture harvests were titrated by the I.S. route, using 10 mice for each serial 10-fold dilution in saline. Fifty percent end-points were calculated by the method of Reed and Muench and the results expressed as titer per 1 ml of tissue culture supernatant. Parallel tissue culture titrations were performed in monocellular layer kidney roller tubes with Type 2 strains from the same tissue culture passage material used for inoculation of mice. Tenfold serial dilutions were made in Hanks' balanced salt solution (without bicarbonate), and 0.1 ml inoculated into each of 2 tubes, which were observed for cytopathogenic effect for 7 days. Results are expressed as titer per 1 ml of fluid.

Results. Isolations in mice. A. *Types 1 and 3.* Fifty-one Type 1 and 20 Type 3 isolates were inoculated into mice I.S. In view of the entirely negative results, as shown in Table I, I.C. inoculation of mice and titrations in tissue culture was not performed. B. *Type 2.* Harvests from the first or second passage in kidney tissue culture of Type 2 viruses from 56 patients produced paralysis in mice by the I.S. route in 45 instances (80%). (Table I) The average incubation period to paralysis of the first mouse in all groups receiving the undiluted tissue culture virus was 3.8 days.

These same 56 tissue culture passage fluids were also inoculated into mice by the I.C. route and only 11 (20%) were positive. Ten produced paralysis in only one or 2 mice out of 10, and one paralyzed all 10 mice inoculated. The average incubation period to first symptoms was 7.5 days (including one group in which the single positive mouse became paralyzed on the 27th day).

Direct inoculation of stool suspensions into mice. Thirty-nine stool suspensions were

tested intraspinally. As shown in Table I, direct isolation of Type 2 strains was possible with 6 (15%), although no more than one or 2 animals of 10 inoculated developed paralysis. Virus concentration in the stool suspensions was not determined by titration in tissue culture. However, no correlation was apparent between ease of isolation directly from the stools and the titer of first passage tissue culture fluid either in mice or in tissue culture. All positive isolations directly from stool were from specimens which were also positive in mice after tissue culture passage. Six stools, positive by the I.S. route, were tested intracerebrally. One produced paralysis in a single mouse. The identity of this isolate was proved by specific neutralization with Type 2 serum.

Relationship between I.S. titer and I.C. results in mice with Type 2 isolates. Table II shows a comparison of the I.S. and I.C. routes of inoculation. No fluid was positive by the latter which had not produced paralysis by the former. A correlation between I.S. titer and I.C. pathogenicity is suggested by the fact that all 8 samples with I.S. titers greater than 10^{-4} were positive by the I.C. route, whereas only one of 18 with titers less than 10^{-2} was similarly positive. The one strain that paralyzed all mice inoculated I.C. titered $10^{-5.5}$ by the I.S. route.

Relationship between titers in tissue culture and I.S. in mice. In Table III the pathogenicity for monkey epithelium cells as indicated by titer in tissue culture and for the mouse spinal cord as evidenced by the I.S. titer is compared. There appears to be no significant correlation between the two since at any level of pathogenicity for one system there is a wide spread of the titer end-points

TABLE II. Relationship between I.S. Titer and I.C. Results with Type 2 Strains.

I.S. titer	I.C. results	
	Positive	Negative
>4*	8	0
3-4	1	9
2-3	1	8
<2	1	17
0	0	11
Total	11	45

* Reciprocal of log titer/ml.

TABLE III. Comparison of Tissue Culture and Mouse I.S. Titers with Type 2 Strains.

T.C. titer	I.S. titer				Total
	Neg. (undil.)	<2	2-3	>4	
>7	3	2	1	0	10
6-7	5	7	4	7	26
5-6	2	7	2	3	15
4-5	1	2	2	0	5
Total	11	18	9	10	56

All titers expressed as the reciprocal of the log titer/ml.

by the other. Both first and second passage harvests from kidney roller tubes were tested in mice with ten strains. With 3, both were positive in high titer by the I.S. route. Of the remaining 7, 4 converted from negative to positive, but with low titers; 2 remained negative. Again, no correlation between tissue culture titer and the results in mice was demonstrable.

Relationship of clinical type of disease in patient source of virus and pathogenicity of strains. There were insufficient numbers of Type 2 virus strains from non-paralytic(11) and minor illness(8) patients to allow any significant comparison of titer results between these groups and the larger group of paralytic cases. However, while there appeared to be no difference in the tissue culture titers between these 3 groups, the strains from minor illness patients tended to have lower pathogenicity for mice than those from paralytic and non-paralytic cases.

Discussion. The entirely negative results obtained in the present study with 51 Type 1 and 20 Type 3 strains confirm the fact that the mouse pathogenicity of these immunologic types is extremely low.

Contrary to the difficulties encountered by earlier workers in adapting strains of Type 2 polio virus to mice by the I.C. route, the present study demonstrates the ease with which this can be accomplished with recently isolated strains when mice are inoculated I.S. Of a total of 56 strains tested by both routes, 20% were positive I.C., while 80% produced paralysis by the I.S. route. In a previous publication, one of the authors(16) showed a difference of approximately a log in titer between the two routes. In the present study all

strains with I.S. titers greater than 10^{-4} per ml produced paralysis by the I.C. route, while only 8% of those with I.S. titers less than 10^{-4} were similarly positive.

That the high proportion of positive results reflects increased susceptibility of mice to I.S. inoculation, rather than increased concentrations of virus obtained in tissue culture, is brought out by the results of parallel titrations in the two systems. No correlation between tissue culture and mouse titer could be demonstrated. Four of five isolates in which the tissue culture titer was less than 10^{-5} produced paralysis in mice. On the other hand, of 36 fluids with tissue culture titers greater than 10^{-6} , eight were entirely negative in mice.

It is of some interest to note that direct primary isolation was possible in mice with 6 of 39 stool suspensions tested I.S., and that in one case, primary isolation was accomplished by the I.C. route. Five were obtained from paralytic cases, and one (that positive I.C.) from a non-paralytic case.

Numbers of specimens available from different classes of clinical disease were too small for significant comparisons, but the Type 2 strains isolated from those with minor illness tended to be low in their pathogenicity for mice by the I.S. route. Further investigation of this relationship between severity of clinical disease and pathogenicity must await the availability of larger numbers of strains.

Summary. Polio virus in tissue culture fluids from first or second passage in monkey kidney roller tubes was tested in mice. Fifty-one Type 1 and 20 Type 3 strains were entirely negative when inoculated I.S. Forty-five of 56 Type 2 strains produced paralysis by the I.S. route, and 11 by the I.C. route. Those having the higher titers by I.S. route tended to be positive I.C. No correlation between tissue culture titer and mouse I.S. titer was apparent. Six strains of Type 2 were isolated directly in mice from 39 stools tested.

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Occurrence of Pantothenyl Cysteine in Natural Materials.* (22127)

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Pantothenyl cysteine (PA-cysteine)[†] has been recognized as an early intermediate in the biosynthesis of coenzyme A (CoA) from pantothenic acid (PA). A liver enzyme can convert PA-cysteine to pantetheine (LBF) (1). PA-cysteine has been shown to be a precursor of LBF in *Acetobacter suboxydans* and it is strongly stimulatory to this organism (2,3). It is of interest to determine whether PA-cysteine occurs naturally. Because of the need for specificity and ability to determine materials in small quantities, a bioautographic method was developed to determine various conjugates occurring in natural materials. A strain of *A. suboxydans* 621 was used. This bacterium will grow on most known PA conjugates, and is generally more sensitive to them than to free PA (2,4).

Methods. A synthetic medium previously described (5) with 2% agar was used for the

bioautograph. The sterilized medium was inoculated with a heavy culture of *A. suboxydans* at 40° and poured into a sterile container to a depth of 6-8 mm. This container consisted of a 19 x 50 cm glass plate, which rested within a stainless steel frame 15 mm high. After agar had solidified, a developed paper chromatogram of PA conjugates was sterilized by treatment with ether, and then spread on agar surface. After 10 minutes, the paper was peeled off, agar plate covered with a sterile 19x50 cm glass plate and incubated at 30° for 24 hours. The top plate was then removed and agar surface sprayed lightly with a mixture of PA (10 γ /ml) and yeast extract (100 mg/ml). The glass plate was restored and the whole assembly again incubated at 30° for 24 hours. Aseptic conditions were preserved throughout. The bioautograph was then observed against a background of diffuse light. The PA-yeast extract mixture was introduced because *A. suboxydans* otherwise grows very slowly under conditions employed. This mixture stimulated growth throughout the entire agar plate but it exerted its greatest effect in areas where growth had already been initiated, i.e., where prior contact had been made with the PA conjugates on the developed chromatogram. A beef liver preparation was used as source of naturally occurring PA-cysteine. It was prepared as follows: 15 g of cold beef liver were ground in a Waring blender with 50 ml

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† The following abbreviations will be used throughout this paper: pantothenic acid = PA; coenzyme A = CoA; pantetheine = *Lactobacillus bulgaricus* factor = LBF; pantetheine- γ -phosphate = LBF- γ -phosphate; pantothenyl cysteine = PA-cysteine.

of ice water. This was poured into 50 ml of boiling water and the liquid decanted from the coagulated protein. Trichloroacetic acid was added to 1% concentration and the solution centrifuged. This yellow liquid was then extracted with ether in a liquid-liquid extractor, and the aqueous layer again centrifuged. The supernatant liquid was passed through a Dowex 1 column (2% cross linked), which had previously been charged with 3 M ammonium formate and washed with water. The column was eluted with formate buffer, prepared by adding 90% formic acid to 0.3 M ammonium formate to pH 3.5. The initial 20 ml of eluate (which was presumably free of coenzyme A(6) as well as other phosphorylated PA derivatives) was selected as the source of natural material used on paper chromatograms. Formate was eliminated by adding excess of HCl and concentrating eluate over KOH in vacuum desiccator. This eluate was reduced, first with Zn-HCl, then with H₂S at pH 7.5, to insure that all PA conjugates present were in the thiol form. About 20 μ l of solution were then placed on Whatman No. 1 paper and chromatographed by the ascending technic. 1 γ of PA, LBF, CoA, LBF- γ -phosphate, and PA-cysteine each were used in 20 μ l solution, to give zones of reference on chromatogram. To make sure that no reoxidation to the disulfide took place, some chromatograms were run under a nitrogen atmosphere. Results were identical to those obtained with chromatograms run in air.

Results. This method is adequate for detection and identification of low concentrations of PA conjugates. PA, CoA, and PA-cysteine have been detected at 0.2 γ . Since *A. suboxydans* did not grow on PA phosphates (7), it is not expected that this method will detect all possible conjugates of PA.

Table I summarizes the R_F values of standard conjugates and liver preparation in a number of solvent systems. Pyridine-water-isopropyl alcohol (1:1:1), phenol-water (4:1), or acetone-water-urea (59.7:39.8:0.5) proved to be good systems for distinguishing

TABLE I. R_F Values of Some PA Conjugates, Determined by Bioautographic Technic.

Compound	Solvent			
	Phenol-water (4:1)	Butanol sat. with H ₂ O	Pyridine-isopropyl alcohol-water (1:1:1)	Acetone isopropyl water 59.7%, 39.8%, urea 0.5%
PA-cysteine	.48	.17	.75	.76
PA	.75	.35	.80	.66
CoA	.20	0		.16
LBF- γ -phosphate	.94	.81	.96	.46
LBF	.90	.94	.90	.92
Beef liver	.73	0	.80	.70
preparation	.61	.29	.73	.63
	.50		.60	.54

PA-cysteine from other conjugates tested. Two-dimensional chromatograms using these solvents disclosed three zones of PA activity. One of these corresponded to the free vitamin, another to PA-cysteine. The third zone did not consistently appear throughout the many experiments performed, and it may be present in a much lower concentration. Using butanol-water, it was not possible to resolve the liver preparation sufficiently to demonstrate PA-cysteine.

Summary. A bioautographic technic has been described for the detection of PA conjugates in natural materials. 0.2 γ of PA, PA-cysteine and CoA can be detected. The occurrence of PA-cysteine in beef liver is indicated by this method.

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Analgetic Property of Vitamin K. (22128)

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(Introduced by A. J. Goldforb.)

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Vitamin K stimulates the liver in order to produce prothrombin. Insufficient amounts of vit. K produce insufficient amounts of prothrombin. From this arises one of the conditions for haemorrhagic diathesis. Vit. K is administered to prevent haemorrhagic diathesis following hypoprothrombinemia, after surgical intervention or, prophylactically, prior to operations.

The other properties of vit. K and Synkavit (diphosphoric acid-ester of 2-methyl-, 4-naphtho-hydrochinon) which is pharmacologically identical with vit. K, have not yet been registered. Vit. K possesses, however, a strong analgetic property which has been observed in patients(1) and experimentally proved in animals(2).

We investigated the analgetic property of vit. K on mice according to the Wolfe-MacDonald method(3), which deals with the registration of thermoalgnesia, as follows:

A glass vessel with double walls between which there is water at a constant temperature of 122°F (50°C) serves for testing the susceptibility of an animal to temperature. If the mouse is put into such a vessel, it will lick its paws after a certain time to mitigate the pain caused by the temperature at the bottom of the vessel. The time elapsing from the moment when the animal is put into the vessel to the moment of licking its paws is measured by chronometer. This period indicates the susceptibility of the animal to pain caused by warmth.

The normal reaction of licking in a series of mice, an average of 9.9 sec. served as control time. The mice were then given vit. K. We took measurements 4 times *i.e.* 30 sec., 1½ hr, 2½ hr, and 3½ hr following administration of vit. K. The results are shown in Fig. 1.

Vitamin K has a decidedly thermoanalgetic property, since the reaction to pain occurs much later than in the control mice. Whereas

the reaction took place after 9.9 sec. in the control mice, the reaction in mice which were administered 2 mg/kg of weight of vit. K occurred after 17 sec. and in mice which were given 4 mg/kg of weight of vit. K after 16.3 sec. Increase of the amount of vit. K does not promote the thermoanalgetic effect but lessens it slightly.

To evaluate these results we investigated the thermoanalgetic effect of morphine by the same method. A series of mice were injected with 3 mg/kg of weight of morphine intraperitoneally and another series with 6 mg/kg of weight. The results are given in Fig. 2.

The results of the thermoanalgetic effect of vit. K in doses of 2 mg/kg of weight and 4 mg/kg of weight have been recorded. The thermoanalgetic effect of vit. K is even stronger than that of morphine. Although the maximal effect of morphine occurs immediately after administration the maximum with vit. K occurs toward the end of the experiment. The reason is the rapidity of resorption. Watery solution of

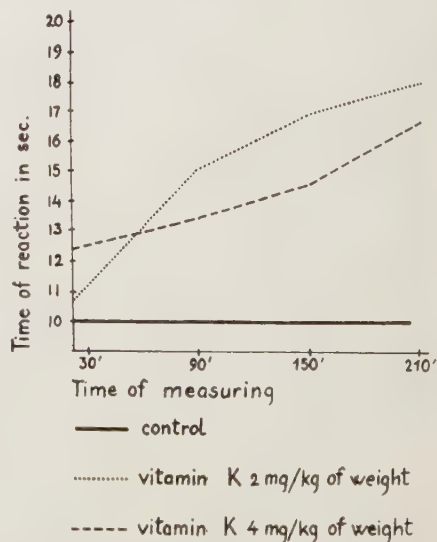


FIG. 1. Effect of Vit. K by normal mouse.

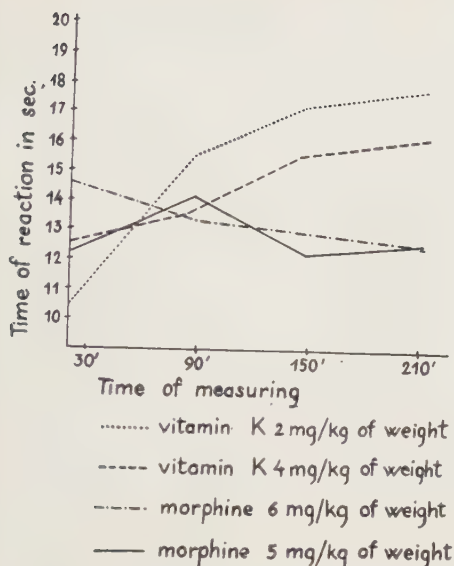


FIG. 2. Effect of Vit. K and morphine.

morphine administered intraperitoneally is readily resorbed while the oil solution of vit. K administered orally is resorbed much slower.

It is known that some substances may elicit the analgetic effect in such a way as to elicit directly or indirectly the mobilisation of cortisone from the root of the suprarenal gland through the hypophysis(4,5). Hench(6) has proved that cortisone has a highly analgetic effect and Jakob(7) has shown that morphine owes its analgetic property to the fact that it may mobilize cortisone. We therefore investigated whether vit. K can mobilize cortisone or some other hormone of the suprarenal gland. We have carried out the above described experiments on suprarenalectomised mice. The results are shown in Fig. 3.

The thermoanalgetic property of vit. K must not be ascribed to its ability to mobilize considerable amounts of cortisone in the organism since the suprarenalectomised mice showed the same reaction as normal mice. From these results we can conclude that the thermoanalgetic property of vit. K may be based on its depressive action either on the cortical or subcortical centres for pain.

To confirm our supposition that vit. K acts on certain areas of the central nervous system in an inhibiting way, we have investigated the registration of mobility according to Ko-

sak's method(8) on a series of mice which were administered vit. K. Kosak's method seemed most satisfactory to us since it secures free movements and registers even the slightest movements. The principle of the method is the following:

The mouse is put into a vessel that is floating on water like a labile boat. All movements are transferred to the vessel and from the vessel recorded on a kymograph. From the frequency of the movements and the size of the registered curves we may deduce objective conclusions on the excitement or immobility of the animal. The results of these experiments are shown in Figs. 4 and 5. Fig. 4 shows the kymographic curve of the mouse which got 4 mg/kg of weight of vit. K and Fig. 5 shows the kymographic curve of a suprarenalectomised mouse given 4 mg/kg of weight of vit. K. From both figures the pronounced sedative and calming effect of vit. K is shown.

We also investigated whether vit. K had an antagonistic property against shock provoked with pentazol and whether it had any influence on the electroepileptic shock.

In a control series the mice were administered 75 mg/kg of weight of pentazol intraperitoneally. All mice displayed typical pronounced convulsions. In a second control series the mice were given 100 mg/kg of weight of pentazol. All mice died shortly after.

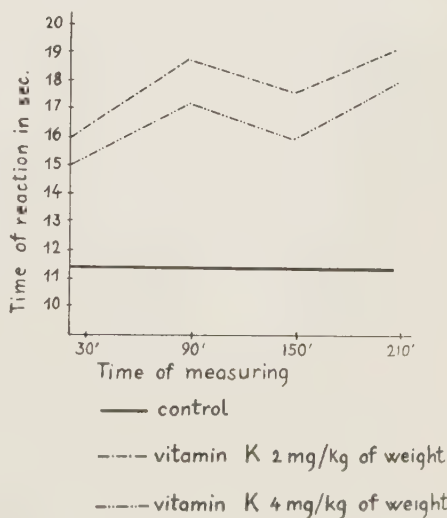


FIG. 3. Effect of Vit. K by suprarenalectomised mouse.

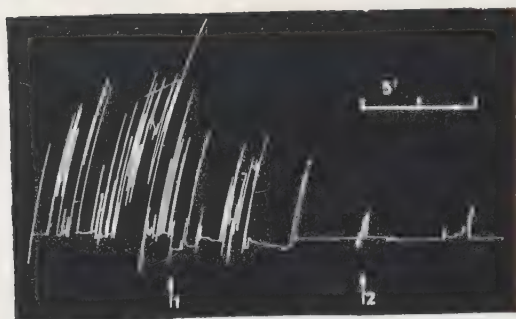


FIG. 4.

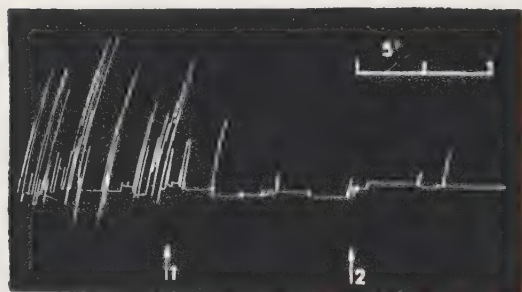


FIG. 5.

Owing to these control results a series of mice were given first 4 mg/kg of weight of vit. K and later 75 mg/kg of weight of pentazol. A second series of mice were first given 4 mg/kg of weight of vit. K and then 100 mg/kg of weight of pentazol. Vit. K had no effect either on the convulsions or on the lethality.

Finally we investigated whether vit. K had any influence on electroshock. The experiments showed that vit. K had no influence either on the intensity or on the duration of the electroshock. We have therefore concluded that vit. K has no depressive action on the motoric sphere of the brain or on the subcortical tonic centers.

We tested the analgetic property of vit. K in 115 patients with inoperative carcinomas

or sarcomas with multiple metastases. All of them were given daily morphine and barbiturates against intense pain prior to initial administration of vit. K. We stopped administering morphine and barbiturates and gave daily injections of 20-30 mg of vit. K. Full analgesia was produced in 95 patients *i.e.* in 75%. In the remaining 20 patients the analgetic effect was either slighter or there was none at all.

Conclusion. 1. Vit. K has shown a considerable thermoanalgetic effect that is also evident in suprarenalectomised animals. This effect is stronger than the thermoanalgetic effect of morphine. 2. Vit. K has shown a strong sedative effect not being due to the mobilisation of the hormones of the suprarenal glands. 3. Vit. K has no depressive property on the cortical or subcortical motor-centers. 4. In patients with inoperative carcinomas and sarcomas with metastases and severe pains, vit. K showed such a pronounced analgetic property that we could cease administration of morphine and barbiturates. The complete analgetic effect was obtained in 73% of cases.

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Anticonvulsant Threshold of CO₂ in Oxygen under High Pressure. (22129)

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Taylor(1) found that addition of small amounts of CO₂ to the breathing mixture hastened the onset of convulsions under high oxygen pressure. Chapin(2) in determining CO₂ toxicity under OHP found no convulsions when mice were subjected to 304 mm Hg ¹CO₂ and 2890 mm or 6080 mm PO₂. This disparity suggested that CO₂ may enhance convulsions only in low concentrations. If this is true it should be possible to determine that tension at which CO₂ becomes anti-convulsant.

Method. A high pressure gas chamber with volume about 1.2 liters was constructed from heavy 4 inch pipe closed at one end. The other end contained a thick lucite window and screwed onto the nipple. A heavy rubber gasket sealed the chamber when tight. Two ports were fitted into the nipple to facilitate introduction and expulsion of gas. Test animals were placed in the chamber which was then closed and ventilated with the test gas. When air had been expelled, exit port was closed and pressure increased to desired value.

Mice were exposed to gas mixtures containing 27, 61, 69, 97, 101, 120, 125, 132, and 165 mm ¹CO₂ each in 3800 mm PO₂. Exposure time to onset of first convulsion was recorded in those cases where a full tonic convulsion occurred.

Results. These experiments confirm the reported enhancement of onset of convulsions by low CO₂, but in addition shows that the time by which convulsions are hastened is about the same when the ¹CO₂ is between 27 mm and 120 mm Hg.

The tension at which only half of the mice convulse and the other half goes on to die without convulsion is about 120 mm ¹CO₂. Table I shows the tabulated results of these experiments.

TABLE I. Influence of CO₂ on OHP Convulsions and Convulsion Time. PO₂ = 3800.

¹ CO ₂	No. used	No. convulsing	Min. exposure before convulsions
0	15	15	21 (10-35)
27	4	4	8 (6- 9)
61	15	15	8 (6-11)
97	3	3	12 (10-14)
101	4	4	9 (8-11)
120	10	5	9 (8-10)
125	4	0	—
132	4	0	—
165	9	1	10

Discussion. This study confirms Taylor's (1) findings that: 1) Carbon dioxide hastens onset of OHP convulsions, 2) within certain limits tension of CO₂ used to shorten convulsion time, appears to be immaterial.

It further appears that CO₂ hastens onset of OHP convulsions in one range and inhibits them in another. The division between these ranges appears to be about 120 mm ¹CO₂. This value falls slightly below that found by Chapin(4) to produce maximal ventilatory response in the hamster and may be related to CO₂ tension at which anesthetic effects are first manifested.

Taylor did not mention failure to convulse in any of his 20 trials although he exposed one cat to 12.2% CO₂ at 55 lb pressure. (He did not specify whether this is gauge or total pressure. Neither did he specify whether this was the pressure due to O₂ and CO₂ together or O₂ alone. If it is total pressure and due to both gases, the ¹CO₂ was 347 mm. If it is gauge pressure and due to both gases, the ¹CO₂ was 439 mm.) This is well within the range for which CO₂ was found by this study to be anti-convulsant.

Stein and Sonnenschein(3) contended that they were unable to confirm the potentiating action of CO₂ on OHP convulsions in cats even though they used CO₂ as low as 1%. Their gauge pressure appears to have been either 75 or 90 lb. The ¹CO₂ at which they failed to get convulsions but only depression

* The author wishes to state his appreciation for the technical assistance of C. and N. Morris, E. Lap-
pat, and I. Miller.

may be calculated at 46 or 54 mm. This is far below the anti-convulsant threshold determined in mice by this study.

Summary. 1. A series of 68 mice was exposed to 5 atmospheres of oxygen in a small pressure chamber. Some of these received pure oxygen while others received mixtures of oxygen diluted with varying quantities of CO_2 . All mice receiving only pure oxygen convulsed. All mice receiving less than 120 mm PCO_2 in OHP convulsed. One half of the mice receiving 120 mm PCO_2 in OHP convulsed. The other half went on to die without convulsions. Only one out of 17 mice receiving over 120 mm PCO_2 convulsed. 2. Mice receiving oxy-

gen diluted with CO_2 convulsed sooner than mice receiving pure oxygen. Amount of shortening of convulsion time did not appear to be dependent upon amount of CO_2 diluting the oxygen as long as PCO_2 lay in the range of 27-120 mm. Both of these findings confirm those of Taylor(1). The tension at which CO_2 becomes anticonvulsant appears to be about 120 mm. or about 16% of 1 atmosphere.

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3. Stein, S. M., and Sonnenschein, R. R., *J. Aviation Med.*, 1950, v21, 401.
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Effects of Altitude, Acidosis, and Alkalosis upon Hypoxic Threshold.* (22130)

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(Introduced by C. A. Maaske.)

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Hoff and Breckenridge(1) have contended since acclimatized subjects exhibit hyperventilation at all altitudes above sea level that in acclimatized breathing there is no anoxic threshold. Rahn and Otis(2) on the other hand suggest that the alveolar PCO_2 begins to fall (and the ventilation begins to rise) in all residents of altitude when the PAO_2 is lowered to about 100 mm Hg.

This study was undertaken to determine whether there was or was not a hypoxic threshold for altitude dwellers, if so where it was, and whether it was the same no matter what altitude was represented.

Method. Experiments were carried out on 6 subjects, residents of Denver, Colo. (5300 feet) and on 5 others, 4 of whom had spent 2 months and one who had spent 1 month living on Mt. Evans, Colorado (14,150 feet). Subjects rested on a cot throughout experiment.

* This study was supported in part by funds provided under contract AF 18(600)-1257 with the USAF School of Aviation Medicine, Randolph Field, Texas.

After a 10 minute period to bring them to a resting state they breathed a gas mixture containing 50% oxygen in nitrogen so the ventilation changes incident to breathing oxygen mixtures described by Chapin(3) would not be introduced into the critical phase of experiment. Following this phase subjects immediately switched to rebreathing from calibrated Collins 13.5 L recording spirometer equipped with soda lime CO_2 absorber and initial gas mixture of 50% O_2 in N_2 at Mt. Evans and 35% O_2 in N_2 at Denver. By this method inspired PO_2 was gradually reduced. Terminal gas (Scholander) analyses showed no CO_2 in spirometer at end of experiment. Spirometer O_2 was analysed each minute by pulling small samples through a Beckman infrared oxygen analyzer. The point at which ventilation began to increase was taken as hypoxic threshold.[†] Since, in some respects, chronic NH_4Cl ingestion simulates altitude acclimatization, we thought that testing the effect of NH_4Cl on hypoxic threshold would be interesting. Five of the Denver subjects ingested 6 g of NH_4Cl

TABLE I. Hypoxic Thresholds in Various Situations.

Situation	Tracheal $P_{I}O_2$ at hypoxic threshold (mm)	Range	No. of subjects
Mt. Evans	179	174-182.5	6
Acidosis at Denver	164	153-185	5
Normal at Denver	127	121-131	5
Alkalosis at Denver	107	103.5-110	3
Normal at sea level	93	(Rahn and Otis)	

daily for 5 days, were tested as described above, and showed elevation in hypoxic threshold to 164 mm. Following this finding it was natural to test the effect of bicarbonate. This was done by 3 subjects who ingested 15-30 g $NaHCO_3$ per day for five days and exhibited an average hypoxic threshold drop to 107 mm which is close to that exhibited by normal subjects acclimatized to sea level.

Results. A definite threshold for Denver residents was found at about 127 mm $P_{I}O_2$. This suggests that most Denverites have slight hypoxic drives since average Denver tracheal air $P_{I}O_2$ is about 122 mm. Mt. Evans residents also exhibited thresholds but these averaged 179 mm $P_{I}O_2$. Since sea level subjects are believed by many to reach the hypoxic threshold only when inspired oxygen falls to 12 to 14%, there appears to be an orderly progression of hypoxic threshold with altitude.

Table I shows the mean hypoxic thresholds in each of 4 experimental situations together with the ranges and number of subjects. In addition a sea level figure from Rahn and Otis is included.

Discussion. One might argue, since the hypoxic threshold varies directly with altitude, that it varies inversely with the $P_{I}O_2$ at which one is acclimatized. However, since NH_4Cl ingestion at Denver results in hyperventila-

tion and slightly elevated $P_{A}O_2$ and at the same time increases the hypoxic threshold, this thesis would not appear to be tenable. It might be further argued that pH determines the threshold. However, since altitude acclimatization probably results in slightly elevated pH while NH_4Cl administration lowers pH somewhat and since they both elevate the hypoxic threshold, this thesis seems untenable. It seems more likely that the hypoxic threshold varies with the resting P_{CO_2} level or some

function thereof. P_{CO_2} is low in individuals acclimatized to altitude and in those in chronic NH_4Cl acidosis. Both of these groups exhibit high thresholds. P_{CO_2} is higher in individuals acclimatized to sea level than in Denverites and it is also high in Denverites receiving bicarbonate. Both of these groups show low hypoxic thresholds.

Still further possibilities remain. Acclimatization of the chemoreceptors to the bicarbonate level of the arterial blood may govern the threshold. The chemoreceptors may be involved only indirectly and the level of their response to anoxia may be set by the P_{CO_2} or long term bicarbonate level of the respiratory center. In some way the volume of ventilation, apart from CO_2 level, might set the threshold. On the other hand, if it is CO_2 or bicarbonate level to which one is acclimatized which determines the threshold for hypoxic response, this test might be a more sensitive measure of the extent of CO_2 acclimatization than those we now have.

Fig. 1 shows a plot of these points on a Fenn CO_2 - O_2 diagram. Inspired tensions have been converted to probable alveolar values and alveolar hypoxic threshold CO_2 tensions have been calculated assuming a steady state and assuming that high oxygen depresses breathing in subjects acclimatized to altitude (3). A hypoxic threshold line has been drawn connecting the threshold values. This line confirms the suggestion that a resident of about four thousand feet would be living at his hypoxic threshold. However an alkalotic individual would be above and an acidotic individual would be below the threshold and the latter would be under some hypoxic ven-

† Although it would be conventional where the threshold occurs at a higher O_2 tension to refer to it as a reduced hypoxic threshold (lower intensity of a liminal stimulus), we feel such usage would be more confusing than to refer to the threshold in terms of the oxygen tension at which it occurs. The above situation, therefore, would be called an increase in hypoxic threshold.

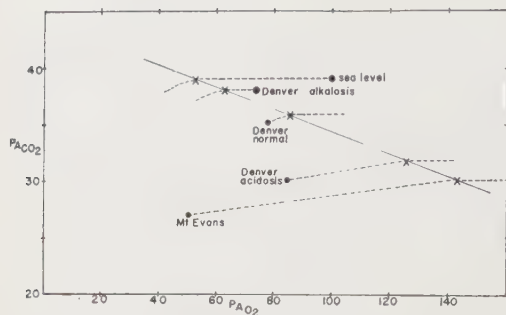


FIG. 1. Hypoxic threshold line is the solid line drawn between probable hypoxic thresholds of residents at sea level, Denver, Mt. Evans, and of Denver residents who are acidotic and alkalotic. Dashed lines indicate probable alveolar gas changes resulting from gradual decreases in alveolar oxygen tensions. When the threshold line is crossed, breathing increases and $P^A\text{CO}_2$ decreases.

tilatory drive.

Summary. 1. Inspired oxygen tensions which produce the first discernible increases in ventilation (hypoxic thresholds) were measured in subjects acclimatized to Mt. Evans, Colo. (14,150 feet) and to Denver, Colo. (5300 feet). In addition thresholds were measured in subjects living in Denver who

were in chronic metabolic acidosis (NH_4Cl) and in chronic metabolic alkalosis (NaHCO_3). 2. Mt. Evans subjects showed an average threshold of 179 mm tracheal $P\text{O}_2$ and those at Denver 127 mm. Reports in the literature pinpoint the threshold for sea level dwellers at about 93 mm. Furthermore the threshold for Denverites ingesting NH_4Cl was 163 mm and for those ingesting NaHCO_3 was 107 mm. 3. Since NH_4Cl changed $P^A\text{O}_2$ and pH in the opposite direction from altitude but changed the hypoxic threshold in the same direction, these agents were ruled out as threshold governors. It was suggested that probably the hypoxic threshold is a function of either the resting $P\text{CO}_2$ or the bicarbonate level to which the individual had acclimatized.

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Prevention and Treatment of Motion Sickness by Intranasal Medication. (22131)

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Rapid absorption of hyoscine hydrobromide (scopolamine) after intranasal instillation has recently been reported(1,2). This route of administration combines simplicity of self-medication with rapid drug absorption. It has the further advantage of circumventing the oral route when nausea, psychic factors, or lack of cooperation make swallowing difficult. This technic should be especially valuable in prevention and treatment of motion sickness. Its effectiveness against swing- and airsickness is the subject of this report.

Experimental. *Scopolamine Spray and Swing Sickness.* Healthy, male adults ranging in age from 17 to 24 years (average 19 years) were used as subjects. The experimental group were given varying amounts of scopolamine in each nostril by a plastic spray bottle† and the remainder were given saline in a similar manner. Exact amount of fluid instilled was determined by loss in weight of spray bottle. Thirty minutes after spraying, the subject was placed in the carriage of a motor-driven swing and swung through an arc of 120° for 20 min-

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† We acknowledge a generous supply by the Doho Chemical Corp.

utes, unless vomiting occurred earlier. Radius of swing was 14 feet and frequency of swingings 14 per minute. Data on occurrence of side effects was obtained from questionnaires distributed upon completion of swinging. Scopolamine was dissolved in 0.9% NaCl solution immediately before use. Originally, it was planned to spray approximately 0.3 mg of scopolamine into each nostril. Therefore, a solution containing 3 mg/ml was prepared and approximately 0.1 ml sprayed into each nostril. The volume sprayed varied considerably and an occasional subject received 1 to 1.5 mg of scopolamine. Dosage level was, therefore, reduced to 2 mg/ml to provide a greater margin of safety. As evident from Table I significant protection was afforded although the average dose was only 0.43 mg ($\sigma = 0.22$) compared with 0.65 mg to 1 mg ordinarily given by mouth. At this dose level, the only side effects markedly increased were those of dizziness, which rose from 23% to over 45%, and dry mouth which was more than twice that of control group. There was no significant increase of drowsiness or blurred vision. Other side effects such as headache, fatigue, nervousness, and sweating were uniformly less than the incidence among the control group. The high percentage of mild dizziness after scopolamine (despite the relatively small dose administered) was disturbing. Therefore, to increase further the margin of safety, scopolamine was prepared in a concentration of 1 mg/ml and administered as before. With this solution, an average dose of 0.26 mg ($\sigma = 0.14$) of scopolamine was sprayed. The number vomiting was slightly less than in the control group but the difference was not statistically significant. Since the detergent sodium lauryl sulfate (Duponal C) increased the absorption of scopolamine in a nasal spray(1) it was added (0.01%) to the saline solution (0.9%) containing 1 mg/ml of scopolamine. As is evident from Table I this preparation sharply decreased the incidence of vomiting from 15.3 to 5.9%. Incidence of dry mouth was greater than normal, but no other significant increases in side effects were apparent. Incidence of headaches, sweating, and fatigue was reduced. The average dose of scopolamine administered was 0.31 mg ($\sigma =$

TABLE I. Effectiveness of Scopolamine against Swing-Sickness when Administered as Nasal Spray.

	No. of subjects	Avg dose, mg	Vomit, %	Protection,† %
Saline control	47		14.9	
2 mg S†	47	.43	4.2*	71.8
Saline control	94		13.8	
1 mg S	94	.26	10.6	23.2
Saline-Duponal control	85		15.3	
1 mg S (0.9%)—Duponal (0.01%) sol.	85	.31	5.9*	61.5

* $P = \leq 0.05$.

† $\frac{\% \text{ of vomiting placebo} - \% \text{ of vomit, medication}}{\% \text{ of vomiting placebo}}$.

‡ S = Scopolamine/ml in saline.

0.12). This was slightly higher than the amount sprayed when Duponal was not employed at the 1-mg dose level.

Scopolamine Spray and Airsickness. The effectiveness of intranasal administration in preventing swing sickness suggested that it be tried therapeutically as well. For this purpose, our subjects were exposed to an hour's flight of standardized turbulence in C-47 (DC-3-type) aircraft. Procedure was the same as previously reported(3) in which the route, altitude, and maneuvers were standardized as rigidly as possible to insure reproducible flights. Originally, the subjects were given the plastic spray bottles containing either control saline-Duponal solution or freshly prepared scopolamine (2 mg/ml) in this solution. Each person was instructed to use the spray as soon as he felt nauseated. It rapidly became apparent, that the subjects were reluctant to medicate themselves since to do so constituted an admission of nausea. Consequently, very few utilized the medication despite obvious distress and subsequent vomiting in many cases. To obviate this difficulty, observers sprayed each subject intranasally 15 minutes after take-off. This time was chosen since in scores of previous flights vomiting rarely occurred earlier, although vasomotor disturbances and nausea were already evident. The spray bottles were weighed before and after use to determine the quantity lost. The subjects were observed for remainder of test period and the incidence of side effects was obtained by questionnaire, as

TABLE II. Therapeutic Effectiveness of Nasal Administration of Scopolamine.*

Dose of scopolamine	No. of subjects	Vomit, %	P†	Protection, %
0 (saline)‡	79	44.3	—	—
.80 ($\sigma = 0.50$)‡	79	22.6	.01	48.6
0 (saline)§	89	28.1	—	—
.2 §	90	17.8	.10-.20	36.6
.4 §	90	15.6	.05	44.5
.6 §	90	10.0	<.01	65.0

* Administered 15 min. after take-off.

† Saline vs medication.

‡ Nasal spray.

§ Nose drops.

already described. The results are summarized in Table II. As can be seen, the scopolamine spray significantly reduced the incidence of vomiting. Since medication was not administered until 15 minutes after flight had begun and the average time of vomiting was 30 to 35 minutes, it is apparent that absorption and action of scopolamine by this route is extremely rapid. The incidence of blurred vision, drowsiness, and dry mouth was increased in the medicated group, although not to the significant level. Sweating was significantly reduced ($P = 0.01$) among those receiving scopolamine.

Scopolamine Nose Drops and Airsickness. Despite careful selection of the spray bottles and indoctrination in their use, the variation in amount of drug given was surprisingly great, ranging from less than 0.10 mg to more than 1.5 mg ($\sigma = 0.50$). To obtain more exact dosage response data, the experiment was repeated utilizing nose drops rather than the spray. Again, 15 minutes after take-off, 0.2 ml of solution was instilled into each nostril. Varying quantities of scopolamine were dissolved in 0.9% NaCl containing 0.01% Duponal so that total dose administered was 0.2, 0.4, or 0.6 mg, respectively. A control group received the Duponal-saline solution alone. The results are given in Table II. Marked protection was afforded by 0.4 and 0.6 mg of scopolamine and apparently some protection even by 0.2 mg. Significant side effects were confined to blurred vision, dry mouth, and sweating. No changes were observed with 0.2 mg of scopolamine, except for a decrease in incidence of sweating. At the 0.4 mg level, blurred vision was reported, and

with 0.6 mg all 3 side effects were accentuated. A slight, but nonsignificant, increase in drowsiness was also reported.

Effectiveness of Scopolamine by Various Routes Against Airsickness. Another practical means for self-administration of drugs is by the sublingual route. Many drugs have been given in this fashion and absorption is known to be rapid. It was of interest, therefore, to compare effectiveness of scopolamine by this route with that after intranasal instillation. Volunteers were again subjected to a period of standardized turbulence in military aircraft before receiving medication. Eighteen minutes after take-off, subjects received a placebo capsule taken by mouth, or 0.65 mg of scopolamine by mouth, or as sublingual tablet. Two minutes later, a fourth group received the same amount of scopolamine in the saline-Duponal solution as nose drops. The 2-minute interval permitted dissolution of the sublingual tablet. Regular 0.01 grain hypodermic tablets were used for this purpose. These small tablets were placed under the tongue and the subject cautioned not to swallow. They were found to dissolve well within the 2-minute period. Exposure time prior to medication was lengthened (20 vs. 15 min) over that in earlier trials to provide a more rigorous test for the drug. As before, scopolamine nose drops markedly lowered the incidence of vomiting (Table III), whereas the drug was completely ineffective when given orally or sublingually. The rapidity of its absorption through the nasal mucosa is also reflected by side effects recorded in Table IV. Nose drops produced highly significant increases ($P = 0.01$) in dizziness, drowsiness, and dry mouth, as well as a significant decrease in incidence of headaches. The only other significant change from the control group was a moderate increase ($P = 0.05$) in

TABLE III. Route of Administration on Effectiveness against Airsickness.

	Dose, mg	Route	No. of subjects	No. of vomit	Vomit, %
Placebo	—	Oral	84	29	34.5
Scopolamine	.65	"	85	30	35.3
	.65	Sublingual	82	26	31.8
	.65	Intranasal	82	13	15.9

TABLE IV. Side Effects of Scopolamine by Various Routes.

Dose, mg	Route	Dizzy	Drowsy	Dry	
				mouth	Headache
0	Oral	16.5	41.2	9.4	22.4
.65	"	23.5	47.1	12.9	16.5
.65	Sublingual	22.4	40.0	20.0*	18.8
.65	Intranasal	45.9†	69.4†	47.1†	8.2†

* P = .05.

† P = .01.

dry mouth among those receiving scopolamine sublingually.

Discussion. It is apparent from these results that intranasal medication is feasible in the prevention and treatment of motion sickness. The rapid action of scopolamine by this route was the most striking feature. In the aircraft, vomiting was largely prevented despite the short period available for its action on an already nauseated individual. Since the drug was not given until 15-20 minutes after take-off and the test period was only 60 minutes, a maximum period of 40-45 minutes was available for absorption and action. As expected because of the short period allowed for absorption, scopolamine by mouth was completely ineffective. Ineffectiveness by sublingual route indicates that absorption here, too, is slower than from the nasal mucosa.

Administration as a spray would seem the simplest technic for intranasal instillation. The marked variation in the quantities nebulized, however, makes this procedure unreliable at present. Small, disposable spray bottles holding only a single dose would obviate

this difficulty, if their production is feasible. For the present, nose drops, although slightly more difficult to administer are preferable since their dose can be more accurately controlled.

Summary. Scopolamine in small doses (0.3-0.4 mg) given intranasally by spray 30 minutes prior to exposure, exerted significant protection against swing sickness. During actual flight testing, addition of a surface active agent (sodium lauryl sulfate-Duponal C) increased its effectiveness. Nasal instillation to subjects 15-20 minutes after take-off sharply reduced the incidence of vomiting from airsickness during subsequent 40-45 minutes. Oral and sublingual administration under these conditions were ineffective. Considerable variations in the drug instilled resulted when given by spray. The use of nose drops allowed more accurate medication. The significance of this mode of administration for treating motion sickness is discussed.

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Changes in Adrenal Medulla Following Treatment with an ACTH-Preparation Containing Carboxymethylcellulose.* (22132)

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Several investigators believed to have produced changes in the adrenal medulla by treatment with certain pituitary preparations. It was thought, for instance, that injections (1) or even oral administration (2) of a special "medullotrophic hormone," as well as parenteral administration of purified somatotrophic hormone (3), can stimulate growth of the chromaffin-cells in adrenals of laboratory rodents. However, none of these changes can be regularly reproduced and definite proof is lacking that any pituitary-preparation exerts a specific effect upon the function or structure of medullary cells. The generally recognized adrenotrophic hormone of the pituitary is called "adrenocorticotrophic" precisely because of its selective effect upon the cortex. We have long been searching for possible medullotrophic actions of ACTH-preparations. We were never able to demonstrate any such effects however, excepting the dilatation of the medullary blood-vessels and a suggestion of hypertrophy in the chromaffin-cells, which were thought to be merely secondary results of the increased blood-flow and the consequent improvement in the nutritional status of the gland as a whole. On the other hand, recently, using the PAS-technic (4), we quite regularly observed definite changes in the adrenal medulla of rats treated for various purposes with certain "long-acting" ACTH-preparations. (5). To illustrate the histologic characteristics of these changes it will suffice to present one such experiment here, since the results were uniformly the same whenever 20 I.U. per day or more was given to rats during 2 weeks or more.

Experimental. In a typical experiment, 20 I.U. of "Duracton" (Nordic Biochemicals Ltd., Montreal) was given subcutaneously, twice daily, to 10 female Sprague-Dawley rats having an average initial body-weight of

100 g. This ACTH-preparation contains 24 I.U./mg, and is made available with carboxymethylcellulose (added as a retarding agent) in an aqueous solution containing 20 I.U./ml. This treatment inhibited growth, so that the treated animals gained only an average of 14 g, while a group of 10 normal control rats gained an average of 42 g during the period of observation. All animals were killed after 14 days, at which time the mean adrenal-weight was 42 mg (range 37-47) in the controls and 92.5 mg (range 90-104) in the ACTH-treated animals. Immediately after autopsy the adrenals were fixed in susa-mixture, embedded in paraffin and stained with hematoxylin-eosin.

The slides again gave the impression that only the cortex participates in the pronounced adrenal enlargement. The individual medullary cells were not conspicuously hyperplastic, although the medullary vessels were markedly dilated. However, slides subsequently stained with the PAS-method revealed numerous rust-colored darker cell-elements throughout the medulla. Some of these cells are elongated or roundish, while others have a typical epithelioid appearance and resemble the adjacent chromaffin-cells. Occasional rust-colored granules were also seen in the cortical cells, especially in the ectopic cell-nests within the medulla, as well as in the cells of the glomerulosa and reticularis layers. Some of the reticulo-endothelial cells, which line the cortical sinusoids, likewise contain such granules (Fig. 1).

The general appearance of many among these cells is reminiscent of mast-cells. We have therefore stained additional sections of these adrenals with such selective mast-cell dyes as neutral red, acridine yellow, toluidine blue and Unna's mixture. The many granules in the mastocytoid cells of the medulla, as well as the individual granules within the cortical cells, stained uniformly, like mast-

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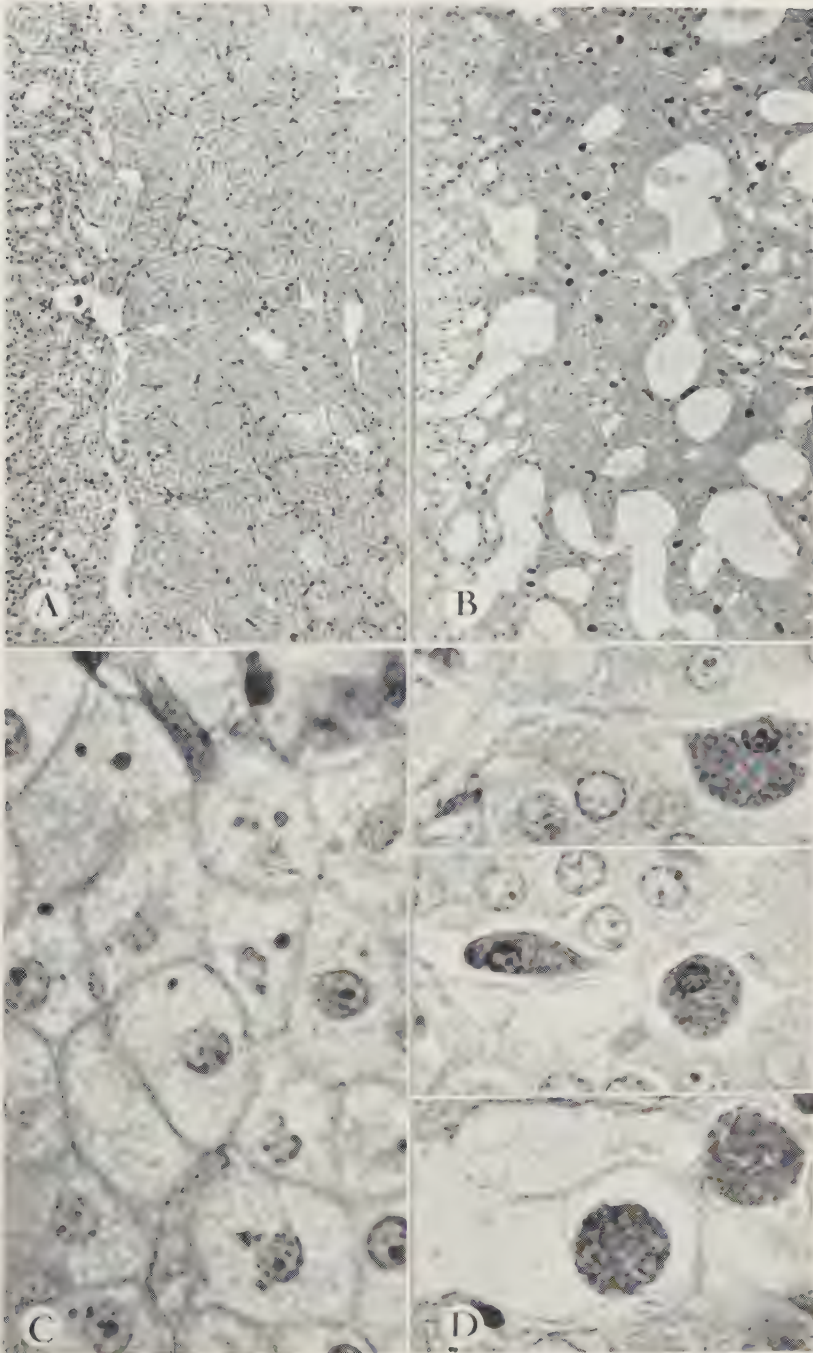


FIG. 1. A. Inner cortex (left) and medulla, under low magnification, in an untreated control rat. B. Same region in an ACTH ("Duraeton") treated rat. Black mastocytoid elements are distributed throughout the chromaffin-tissue of the medulla. The medullary veins are greatly dilated. C. High magnification of a cell-region from the fascicula of an ACTH ("Duraeton") treated rat. Note individual small black granules (which stain like mast-cell granules) in the cortical cells. Their size is usually approximately that of a nucleolus and only a few such granules tend to appear within any one cortical cell. D. Three fields from the medulla shown in "B." Note various types of mastocytoid elements, some of which are epithelioid, some spindle-shaped and some rounded. All the sections in this plate were stained with the PAS-stain, which lends itself particularly well for black-and-white photography.

cells (selectively, but orthochromatically, with neutral red and acridine yellow, metachromatically with Unna's mixture and toluidine blue). Some material with similar staining properties was also found in the blood of the adrenal venules; here it usually adhered to erythrocytes.

A comparison with typical mast-cells was then made by staining sections of the thymus of the same animals on the same slide with their adrenals. It was found that the ordinary mast-cells of the connective tissue in the thymus are smaller, their shape is more regularly ovoid and they contain much smaller and more intensely staining granules than the medullary mastocytoid elements.

Discussion. On the basis of the evidence available to us, it is impossible to advance any hypothesis concerning the possible histogenesis or function of the mastocytoid cells. It should be pointed out however that, after treatment with ACTH-preparations without added carboxymethylcellulose, we have not observed any such mastocytoid elements in the medulla, nor metachromatic granulations in the cortical cells. On the other hand, control experiments with the carboxymethylcel-

lulose-containing solvent alone, gave positive results. Presumably, the mastocytoid elements in the adrenal medulla are due to ingestion by histiocytes of metachromatically staining carboxymethylcellulose granules.

Summary. Observations on rats indicate that treatment with large doses of an ACTH-preparation, to which carboxymethylcellulose was added as a retarding agent, results in the development of mast-cell-like elements within the adrenal medulla. At the same time, in some cortical cells and in adrenal-vein blood, occasional granules appear, which give the characteristic staining reactions of mast-cell granules. Carboxymethylcellulose-solutions containing no ACTH produce similar changes.

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Further Observations on L Forms of *Alpha*-hemolytic Streptococci.*† (22133)

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Isolation of L-type cultures from 2 strains of *alpha*-hemolytic streptococci was described in 1953(1). Many attempts at isolation of

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these forms from *alpha*-hemolytic streptococci in previous years had been unsuccessful. This paper contains the description of two methods which permit with some regularity the finding of strains of *alpha*-hemolytic streptococci which produce L forms. Data are also presented concerning the occurrence of such strains in humans and observations on a pleomorphic strain of *alpha*-hemolytic streptococcus which produced L forms spontaneously without the influence of penicillin.

After the characteristic appearance of L colonies of *alpha*-hemolytic streptococci and the conditions under which they grow became

known, specimens from the human mouth, urethra and vagina were cultivated on appropriate plates. Bacterial growth was inhibited in a part of the plate by penicillin. In cultures made from the mouth, colonies of pleuropneumonia-like organisms usually developed on the inhibited areas and among these occasionally were found much larger colonies which corresponded in appearance to L-type colonies of streptococci. In 3 out of approximately 80 cultures examined, it was possible to subculture these large L-type colonies. They were first subcultured on penicillin plates. When the cultures were allowed to age in the absence of penicillin, *alpha*-hemolytic streptococci appeared in all. Each of these streptococcus cultures produced L-type growth on exposure to penicillin and evidently originated from the reversion of the L forms. The development of L-type colonies corresponding to those of streptococci was not observed in cultures made from the urethra or vagina although streptococcus colonies often developed abundantly on the plates.

These observations were made on the usual horse serum medium containing 0.5% NaCl. After it was observed that on plates containing a high concentration of NaCl or some other electrolytes many *beta*-hemolytic streptococci produced L forms(2,3) it seemed of interest to examine the behavior of *alpha*-hemolytic streptococci on such media.

Preliminary experiments were made by planting tooth scrapings and throat swabs on horse serum agar containing 3% NaCl and exposing part of each plate to penicillin. The development of L colonies of streptococci was observed only rarely on these plates. In a more systematic study made with throat swabs, a mixture of many colonies of *alpha*-hemolytic streptococci from a blood agar plate was inoculated on another plate to check the purity of the culture. From this culture large inocula were transferred to broth and the test plates were then heavily seeded with this broth culture. In this way each test plate received an inoculum originating from a number of colonies found in the same specimen. This procedure was adopted because trials with the descendants of single colonies had remained negative. An increased NaCl con-

tent of the media only rarely favored the development of L-type colonies. However, an increase in the phosphate content either of the sodium or potassium salt, had a marked effect. Abundant development of L-type colonies was observed with concentrations of phosphate between 0.2 and 0.4 M. Only one strain gave a slight growth of L forms with 0.1 M. Concentrations higher than 0.4 M were not tested. Ten out of 40 of such mixed cultures produced L forms on the phosphate plates. Two of the 40 produced L-type colonies with high concentrations of NaCl also, but less abundantly than with the phosphate. The influence of the phosphates was not due to differences in pH as the pH was the same in the chloride and phosphate plates. None of these 40 cultures produced L-type colonies without an increased salt concentration.

Subculture of these L forms was successful only on plates with an increased phosphate content indicating that the high concentration of phosphate ions was important for their growth. The transformation of the cocci into the L forms was probably induced by the penicillin, just as on the regular media. In the case of the strains of *alpha*-hemolytic streptococci which produced L-type colonies on the usual horse serum media, a high phosphate concentration in the medium neither enhanced the transformation of such strains into L forms nor was necessary for the subculture of the L forms.

On the basis of these observations the following procedure can be recommended to obtain L-type cultures of *alpha*-hemolytic streptococci. Plates are made using a tryptic digest of beef heart or beef heart infusion as base, 1.3% agar, and 10% horse serum inactivated at 56°C for 30 minutes. Three ml of a mixture of 20% Na_2HPO_4 and 1.4% KH_2PO_4 is added to 15 ml of the medium. The plates are heavily inoculated as described above. The plates are incubated for 2 hours to allow multiplication of the cocci and then about 1000 units of penicillin are deposited in a small trough cut in the agar. The plates are incubated at 36°C for 4 to 5 days under anaerobic conditions using the Fortner biological method. The behavior of the cultures was consistent. Six strains which originally

produced L forms continued to produce such forms in 4 to 13 subcultures when the transfers were made with heavy inocula. Three to five subcultures of seven strains, which originally did not produce L forms, did not yield this type of growth. The use of an agar base of different composition did not increase the percentage of positive strains.

The observations described indicate that approximately one out of 4 humans harbors in his mouth *alpha*-hemolytic streptococci which, under the conditions employed, produce L-type colonies. Further study has shown that only a few of the single colonies in the throat cultures of such persons will produce L colonies. One throat culture known to be positive was studied more thoroughly and only the descendants of 1 out of 20 single colonies gave positive results. Further subcultures of these 20 colonies behaved similarly. The descendants of negative colonies were always negative. Subcultures obtained by transferring large inocula from a positive strain were always positive. However, only about one-fifth of the descendants of isolated colonies remained positive. This was followed through 4 generations. These observations indicate that not all but only a certain percentage of the cocci in a positive colony transmit to their descendants the ability to produce L forms under the conditions of these experiments. A similar observation was made previously in this laboratory with a Gram positive bacillus(4).

An interesting strain of *alpha*-hemolytic streptococcus was isolated on several occasions from the blood of a patient in the weeks preceding his death.[§] The source of the septicemia was an old kidney abscess; no endocardial lesions were found at autopsy. The organisms were extremely pleomorphic both in the original broth flasks and in transplants on blood agar plates without penicillin. Normal cocci were not observed and the cultures consisted of large round bodies, small granules and long filaments, some with swellings. The

cultures resembled those of *Streptobacillus moniliformis*. The nature of the organisms was established only after several transfers, when the usual coccal form of growth was resumed. When transferred immediately after isolation to horse blood and horse serum agar plates without penicillin and without increased electrolyte concentration, L colonies developed spontaneously among the pleomorphic coccus colonies. When the cultures were exposed to penicillin only L colonies developed.

Discussion. The L forms of bacteria can be recognized at present only by the growth of characteristic colonies. This criterion was used in all cases reported in this paper. The occurrence of large bodies or granules in the cultures, as accepted by several authors,^{||} is not sufficient proof of transformation of bacteria into L forms. Such morphological elements are often present in cultures without signs of multiplication. The expressions "L forms," "L-type growth" and "L cultures" are synonymous and refer to the same fundamental phenomena.

The L-type cultures described in this report undoubtedly originated from *alpha*-hemolytic streptococci. The macroscopical and microscopical appearance of the cultures is characteristic, and differs from that of PPLO which could conceivably contaminate the cultures. The serological specificity of the L forms studied earlier and in the course of the present studies was similar to that of the parent streptococci. The observation that streptococci reappeared in the cultures of L forms and that these cocci reproduced L forms under appropriate conditions is further evidence for the relation between the cocci and these L forms.

The tendency of strains of *alpha*-hemolytic streptococci to produce L forms is as variable as in the other species. The behavior of these strains, as far as the production of L forms is concerned, is in many respects similar to that observed in *Bacteroides*(5). One strain of *alpha*-hemolytic streptococcus produced L forms spontaneously on the usual media. A few strains produced L forms on similar

[§] We are indebted to Lawrence J. Kunz, bacteriologist of the Massachusetts General Hospital, for this strain and many others mentioned in this publication.

^{||} See discussion in (3).

media under the influence of penicillin. High concentrations of NaCl increased slightly and high concentrations of phosphate increased markedly the number of strains which produced L forms. The behavior of the descendants of individual coccal chains of a strain which produced L forms was variable. In some strains the majority of the cocci present in a single colony lost the ability to produce L forms.

It is of interest that in several species such as *Bacteroides* and the hemophylic bacteria, which are the usual inhabitants of the human body, pleomorphism and spontaneous production of L forms is more frequent among the strains isolated from pathological processes than among those isolated from their usual habitat. Similarly, the strain of *alpha*-hemolytic streptococcus which produced L forms spontaneously originated from an abscess of long duration, and was characterized by extreme pleomorphism. This relation cannot be due entirely to the influence of anti-

biotics, because some of these observations were made before they came into use.

Summary. High concentrations of potassium and sodium phosphates incorporated in the media markedly increased the number of strains of *alpha*-hemolytic streptococci which produced L-type growth when exposed to penicillin. High concentrations of NaCl produced this effect only slightly. Using the phosphate media, strains of *alpha*-hemolytic streptococci which produced L forms were found in about one-fourth of the throat cultures examined. One unusual strain of *alpha*-hemolytic streptococcus produced L forms spontaneously without exposure to penicillin.

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Endogenous Formation of Hippuric Acid.* (22134)

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(Introduced by Emil L. Smith.)

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Occurrence of hippuric acid in mammalian urine has been known since its isolation by Liebig(1) in 1829. It has usually been supposed to arise by conjugation of benzoic acid of dietary origin with glycine in the body, and a great part of biochemical investigations of hippuric acid formation have been concerned with glycine metabolism. Recently, presence of hippuric acid in urine of fasting humans was reported and a possible endogenous origin was suggested for it(2). Shortly thereafter, Schreier *et al.*(3) observed a dilution in specific activity of radioactive benzoic acid in

fasting rats, and suggested that an endogenous formation of benzoic acid could explain their results. In addition, they cited other observations, unsupported by experimental data, which were in agreement with this idea. Our investigation indicates that in humans and in the white rat there is a small amount of hippuric acid produced endogenously and that in the rat the aromatic ring of phenylalanine can, to a slight extent, serve as a precursor for the benzoic acid moiety. A major proportion of hippuric acid present in urine of humans and of rats receiving a natural diet is, however, derived from dietary precursors.

Methods. Albino rats (Sprague-Dawley), weighing 300 to 400 g, were fed either Purina

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chow or lactalbumin-dextrin diet which has been described earlier(4). Each animal was allowed 15 g of food daily and given water *ad libitum*; fasting animals were allowed water. For experiments in which a laboratory diet was used with humans, the basal diet contained lactalbumin, sucrose, a vegetable oil (Wesson oil), vitamins and minerals(5). The amounts allowed in g/kg/day were lactalbumin, 1; sucrose, 0.8; fat, 0.8; mineral salts (No. 2, General Biochemicals, Inc.), 0.2, and vitamins. Rat urine was collected with toluene as a preservative; 24-hour samples were acidified to pH 2, extracted twice with 3 volumes of ethyl acetate, and the ethyl acetate was concentrated *in vacuo*. Final volume was adjusted to contain about 0.2 mg of hippuric acid/ml of ethyl acetate. Human urine was collected and frozen immediately; 24-hour samples were assayed for creatinine and organic acids of an aliquot were extracted by the standard procedure reported previously(6). Hippuric acid concentration of the ethyl acetate extracts was estimated by modification of procedure described by Gaffney *et al.*(7). Aliquots which contained between 2 and 10 μ g of hippuric acid were placed on Whatman No. 1 filter paper along with 1 to 10 μ g amounts of authentic hippuric acid, and the chromatograms developed with isopropyl alcohol-aqu. ammonia-H₂O (8:1:1) for 16 hours at 25°C. Hippuric acid ($R_f = 0.59$) was visualized by spraying with *p*-dimethylamino-benzaldehyde/Ac₂O/NaOAc reagent(4) followed by either heating at 100°C for 5 minutes or letting the sprayed chromatograms stand overnight; the latter procedure results in a better background on chromatograms. The amount of hippuric acid was estimated by comparing intensity of color of unknowns and standards as seen with a Kodak cold light X-ray illuminator. Recovery experiments showed that the procedure for rat urine gave a quantitative recovery, and that procedure used for human urine gave approximately 90% recovery of hippuric acid. Accuracy of estimation is within $\pm 20\%$, which is adequate for purposes of these experiments.

The results (Table I) show that a major portion of hippuric acid excreted by rats receiving a chow diet arises from some pre-

TABLE I. Excretion of Hippuric Acid by the Rat.

Regimen	No. of animals	Days studied	Avg daily hippuric acid excreted (mg/kg body wt./day)
Laboratory chow	6	4	22.5 $\pm$ 6.1
Laboratory diet			
Day 1	6	1	14.0 $\pm$ 5.0
2	6	1	8.8 $\pm$ 4.0
3	6	1	5.6 $\pm$ 2.5
4	6	1	5.0 $\pm$ 1.0
5	3	1	4.3 $\pm$.3
6	3	1	3.9 $\pm$.6
Fasting			
Day 7	3	1	4.4 $\pm$ 1.4
8	3	1	3.6 $\pm$.2
9	3	1	3.5 $\pm$.1
10	3	1	3.6 $\pm$.5
11	3	1	3.6 $\pm$.4
12	3	1	3.6 $\pm$.3
13	3	1	3.4 $\pm$.3
Synthetic diet + 2% sulfasuxidine—5th and 6th days	3	2	3.1 $\pm$.3
Synthetic diet + 2% sulfasuxidine + 200 mg L-phenylalanine intraper.	3	3	3.3 $\pm$.2

cursor in the diet; no preformed hippuric acid could be detected in the chow. When animals were given lactalbumin diet, hippuric acid excretion fell during the first few days to 3 to 4 mg/kg/day and then remained constant. Fasting or inclusion of 2% sulfasuxidine in the diet to modify intestinal microorganisms did not lead to significant decrease in hippuric acid excretion nor did oral administration or intraperitoneal injection of phenylalanine lead to increased excretion.

The results obtained with humans (Table II) were similar to those with rats. A much higher excretion of hippuric acid was observed in individuals eating a natural diet than in the same persons after they had eaten a synthetic diet similar to that fed the rats. Excretion of hippuric acid decreased rapidly as soon as synthetic diet was commenced, and then remained relatively constant, with different individuals excreting different amounts, ranging from 1 to about 3 mg/kg body weight/day—values similar to those observed with rats.

Some caution is mandatory when a color reaction is applied to the analysis of materials

TABLE II. Hippuric Acid Excretion in the Human Receiving a Laboratory Diet.

	Hippuric acid excretion (mg/kg/day)					
	Natural diet	Consecutive days of laboratory diet				
		1	2	3	4	5
MA (♂)	14.3	4.4	.7	.5	.7	.7
VP (♂)	7.1	2.3	1.6	1.3	1.5	1.7
PW (♀)	58.0	23.0	3.3	2.4	2.4	2.0

of biological origin. To assure that the material estimated with the reagent of Gaffney *et al.*(7) actually was hippuric acid, the final 24-hour urine sample of one person receiving the synthetic diet for 5 days was extracted in the usual manner, and hippuric acid was isolated from the ethyl acetate extract (200 ml, containing an estimated 100 mg of hippuric acid) with the use of a modified counter-current distribution procedure. Thirty-eight mg of impure hippuric acid, obtained in the first crop, was recrystallized (Norit) from 0.6 ml of hot water to yield 16 mg of white material. The isolated compound was identical with authentic hippuric acid in all its physical properties. The demonstration that hippuric acid actually could be isolated from urine of an individual receiving a well-defined diet supports the conclusion that the material estimated as hippuric acid in this work actually was hippuric acid and not another chromogen with similar chromatographic behavior.

Preparation of 3-C¹⁴-L-phenylalanine. 77.9 mg of 3-C¹⁴-DL-phenylalanine hydrochloride (Tracerlab, Inc., 3.84 Mc/mg) and 227.1 mg of inert DL-phenylalanine were mixed to provide 291.0 mg of DL-phenylalanine (1 Mc/mg). This material was resolved by modification of the method of Wretling(8). 115 mg of L-phenylalanine, $\alpha]_D^{24} = -34^\circ$ (c,l,H₂O) (-35°)(8), and 101 mg of D-phenylalanine, $\alpha]_D^{24} = +33^\circ$ (c,l,H₂O) were obtained.

Isolation of labelled hippuric acid from rat urine. Hippuric acid was extracted from 24-hour samples of urine with ethyl acetate as described above, and a small aliquot used for estimating total quantity present. Ethyl acetate solution was then extracted 4 times with small portions of saturated NaHCO₃ solution and 30 mg of carrier hippuric acid was added to the combined bicarbonate extracts. The

solution was cooled to 0°, acidified to pH 2 by the addition of 6 N HCl, and allowed to stand overnight in a refrigerator. The hippuric acid was recrystallized from hot water. Hippuric acid, obtained by the above procedure, was hydrolyzed by heating with 2 ml of 6 N HCl at 110° in a sealed tube for 12 hours. The acidic solution was extracted 3 times with 5 ml portions of ether, the combined ether extracts were evaporated to dryness, and benzoic acid was purified by sublimation at 120°; m.p., 122°. For counting, aliquots of urine, of the ethyl acetate extract, and ethyl acetate solutions of hippuric and benzoic acids were placed on aluminum discs (1-inch diameter). Radioactivity was measured on duplicate samples with an internal gas flow counter having a geometry of about 50%. Sufficient counts were measured to allow a counting error of $\pm 5\%$ and the counts were corrected for background and self-absorption. The results of tracer experiments with 3-C¹⁴-L-phenylalanine and uniformly labelled C¹⁴-glucose are given in Table III.

Discussion. The experiments reported here serve to establish 2 points: first, there is a small endogenous production of benzoic acid in rat and human, and second, this benzoic acid probably is formed in some indirect and, as yet unknown, manner from phenylalanine.

By far the greater proportion of the hippuric acid excreted by rats and humans who are ingesting a natural diet has its origin in dietary precursors—either combined benzoic acid present in plant materials or, in the case of humans, additional small amounts of sodium benzoate, which is added to some foods as a preservative. After the institution of well-defined laboratory diet, amount of hippuric acid excreted in urine of both species decreased rapidly over 2 to 3 days to a fraction of the amount observed originally. The amount, on a body weight basis, of this endogenous hippuric acid excreted was comparable in human and rat.

In the earlier report of presence of hippuric acid in urine of a fasting human(2), the amount of hippuric acid excreted was somewhat greater than that observed here. This may have resulted because of too short a

TABLE III. Results of Tracer Experiments with Rats.

Compound administered by intraper. inj. →	¹⁴ C-Glucose; one portion		3- ¹⁴ C-L-Phenylalanine; 3 portions at 6 hr intervals		3- ¹⁴ C-L-Phenylalanine; one portion		
Days studied →	Fasting 2nd	3rd	Laboratory diet 6th	7th	Fasting 2nd	3rd	4th
Amt of compound given (mg)	65.5		1.		1.		
¹⁴ C given (cts/min. × 10 ⁻⁸)	.95		.90		1.		
¹⁴ C in 24 hr urine (cts/min.)	7530	990	4610	1760	8790	4370	3000
Proportion of ¹⁴ C excreted in urine (%)	.793	.103	.512	.195	.879	.437	.30
¹⁴ C in EtOAc extract (cts/min.)	870	380	1760	—	7970	3330	2980
Proportion of urinary ¹⁴ C in EtOAc (%)	11.5	38.5	38.2	—	90.6	76.2	99.4
Hippuric acid excreted (mg)	1.0	1.	1.	1.	1.	1.	1.
Hippuric acid isolated (30 mg carrier added) (mg)	22.	—	23.7	—	24.	—	—
Sp. act. of isolated hippuric acid (cts/min./mg)	0	—	17.5	6.9	20.1	9.5	7.
Sp. act. of benzoic acid from isolated hippuric acid (cts/min./mg)	—	—	14.8	—	17.4	—	—
¹⁴ C in benzoic acid portion of hippuric acid (cts/min.)	0	—	459	—	540	—	—
Proportion of ¹⁴ C excreted in benzoic acid moiety of hip- puric acid (%)	0	—	0.051	—	0.054	—	—

period of fasting before urine was collected; the data of Table I show that a constant low excretion of hippuric acid was not observed until the third day of ingestion of laboratory diet. Presumably, this length of time is required for material of dietary origin present in intestinal tract to be cleared from the body. A similar finding was made in connection with excretion of *m*-hydroxy-hippuric acid, which is also derived in large part from some dietary precursor(6).

The tracer experiments with ¹⁴C-labelled phenylalanine, designed to test the possibility that benzoic acid might arise directly from phenylalanine by removal of the 2 terminal carbon atoms of the side chain, clearly showed that, although phenylalanine may definitely serve as precursor to a very limited extent, it can hardly be a direct precursor. The very small amount of radioactivity of administered 3-¹⁴C-phenylalanine appearing in hippuric acid excreted was present in the benzoic acid portion of the hippuric acid rather than in the glycine portion, in which it might have occurred as the result of glycine formation from

acetoacetate or acetate formed by complete breakdown of phenylalanine. The possibility that *de novo* synthesis of a small amount of ¹⁴C-containing benzoic acid from acetate might have been carried out by intestinal microorganisms was considered and was tested by administration of uniformly labelled ¹⁴C-glucose, which has been shown to serve as precursor for microbial synthesis of aromatic rings. Hippuric acid excreted after administration of labelled glucose did not contain a detectable amount of ¹⁴C.

Thus, the observation of a significant production of endogenous benzoic acid in the rat, which was indicated by the dilution of administered ¹⁴C-benzoic acid in the work of Schreier *et al.*(3), has been confirmed in the present experiments. In addition, it has been shown that phenylalanine serves but poorly as precursor, and is probably first converted to some unknown intermediate which gives rise to benzoic acid. Utilization of 3-¹⁴C rather than ring-labelled phenylalanine leaves open the possibility that phenylalanine might have served as precursor for ¹⁴C-benzoic acid,

which then underwent decarboxylation and re-carboxylation, losing the label in the process. In addition to absence of known metabolic transformations of this type, however, lack of incorporation of C^{14} from C^{14} -labelled glucose into the hippuric acid renders this possibility unlikely.

Summary. 1. Humans and rats receiving a purified laboratory diet excrete hippuric acid, 1 to 3 mg/kg/day. This amount appears to be formed endogenously and its excretion in the rat is not affected by fasting, by inclusion of sulfasuxidine in the diet, or by injection of phenylalanine. 2. 0.05% of the radioactivity of 3- C^{14} -L-phenylalanine injected into a rat was excreted in 24 hours in the form of hippuric acid. Further experiments suggested that endogenous hippuric acid could not be de-

rived from phenylalanine in any direct manner.

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Studies on Thrombin Formation. (22135)

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Treatment of plasma with barium sulfate has been reported to remove the following clotting factors: Prothrombin, the serum prothrombin accelerator system*(1), plasma thromboplastin component*(2) and plasma thromboplastin*(3). However, antihemophilic factor* (AHF) and plasma-accelerator-globulin* (PacG) are supposedly not removed by the adsorbent(4). The present study is concerned with the influence of AHF and of PacG on the rate of thrombin formation in a reaction mixture containing calcium ions and platelets as well as factors removed from bovine plasma by barium sulfate (plasma eluate). Concomitant investigations were made on the influence of a barium sulfate eluate from rabbit serum (serum eluate) on the rate of thrombin formation.

Materials. *Bovine plasma:* Oxalated bovine blood (9 parts of blood and 1 part of

3.7% potassium oxalate in 1% oxalic acid) was collected at the slaughter house and centrifuged at 4°C. *Veronal buffer:* A stock solution was made by dissolving 19.428 g sodium acetate • 3H₂O and 29.428 g sodium diethylbarbiturate in one l of distilled water. Veronal buffer of pH 7.2 was used throughout and consisted of 100 ml of the stock solution, 40 ml of 8.5% sodium chloride, 100 ml of 0.1 M hydrochloric acid and 260 ml of distilled water. *Bovine platelets* were obtained from oxalated bovine plasma by differential centrifugation according to the procedure described by Seegers and his associates(5). After the final washing with 0.9% NaCl, the platelets were dried with acetone and then pulverized in a mortar. Suspensions of the dried platelet material were made in veronal buffer. *Bovine plasma eluate:* Oxalated bovine plasma was stirred at room temperature for 10 minutes with BaSO₄ (50 mg/ml), centrifuged at 4°C and the supernatant plasma decanted. The BaSO₄ was washed successively with 0.9% NaCl, 0.1 M sodium oxal-

* For a different terminology of these factors reference is made to Appendix, The Coagulation of Blood. Method of Study. Edited by L. M. Tocantins, Grune and Stratton, N. Y. City, 1955.

ate, and 0.9% NaCl, the volumes of each washing being equal to the original plasma volume. The BaSO_4 was then eluted twice with 0.1 M sodium citrate solution (1/10 of the original plasma volume) by stirring for 10 minutes at room temperature, and then centrifuging and decanting. The two citrate eluates were combined and dialyzed against distilled water at 4°C until free of citrate and then lyophilized. A given amount of the dried preparation was dissolved in veronal buffer, corresponding to definite plasma concentrations. *Rabbit serum eluate* was prepared from stored aged rabbit serum of low prothrombin content according to the procedure described for the plasma eluate. An amount of the dried material was extracted with veronal buffer equivalent to serum concentration, centrifuged for 20 minutes at 4°C and the clear supernatant was employed in the various tests. *Plasma accelerator globulin (PACG)* was prepared according to the method of Lewis and Ware(6) from bovine oxalated plasma after treatment with BaSO_4 . The final preparation was dissolved in a volume of 0.9% NaCl, corresponding approximately to 1/5 of the original plasma volume, and kept frozen in small aliquots until used. *Antihemophilic globulin factor (AHF)* was prepared according to the procedure described by Brinkhous and his associates(7,8). BaSO_4 -treated oxalated bovine plasma was heated for 10 minutes at 52°C to remove fibrinogen. After centrifugation, ammonium sulfate was added to the supernatant to 25% saturation. After one hour at 4°C, the resulting precipitate was removed by centrifugation, dissolved in distilled water and dialyzed at 4°C against 0.9% NaCl. Final volume of the AHF solution was adjusted to the original plasma volume, and kept frozen in small aliquots until used. *Fibrinogen* was prepared from oxalated bovine plasma according to the method of Seegers and his associates(9). Before dilution, the fibrinogen solution was treated with BaSO_4 (100 mg/ml) to remove any absorbable clotting factors which may have been present. For the test, the fibrinogen was diluted with 0.9% NaCl to a concentration of 0.4%. The fibrinogen

preparation contained 89% clottable protein.

Methods. *A. One-stage thrombin formation procedure:* The total incubation mixture consisted of 1.25 ml plasma eluate (2 times plasma concentration) in veronal buffer, 0.6 ml AHF solution, 0.6 ml PACG solution, 0.55 ml serum eluate (serum concentration) in veronal buffer, 0.15 ml platelets suspension (1.5 mg/ml veronal buffer), and 0.3 ml CaCl_2 (0.125 M). Veronal buffer was substituted for one or more of the above reagents in the various experiments. On the addition of the CaCl_2 to the other factors and agitation of the mixture, a stop watch was started and the reaction mixture was incubated at 26°C. Thrombin formation was determined at timed intervals by the addition of 0.1 ml of the reaction mixture to a fibrinogen solution (0.3 ml veronal buffer and 0.1 ml fibrinogen) at 37°C. When the clotting times (as obtained by the tilt method) were below 12 seconds, the 0.1 ml portions of the reaction mixture were suitably diluted with veronal buffer before addition to the fibrinogen solution. The recorded clotting times were interpreted in terms of National Institutes of Health (NIH) thrombin units from a curve prepared with a standard NIH thrombin preparation.† *B. Two-stage thrombin formation procedure:* In

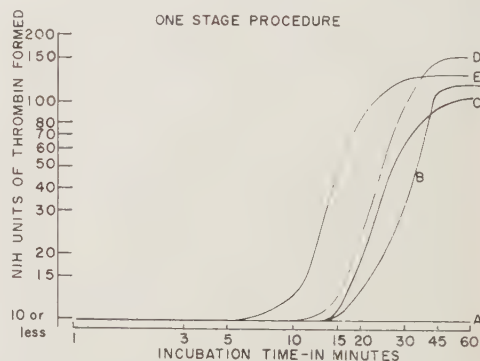


FIG. 1. Formation of thrombin in bovine plasma eluate, platelets and CaCl_2 in the presence of serum eluate, AHF, PACG and AHF plus PACG. A, plasma eluate, platelets and CaCl_2 ; B, plasma eluate, platelets, CaCl_2 , and serum eluate; C, plasma eluate, platelets, CaCl_2 and AHF; D, plasma eluate, platelets, CaCl_2 and PACG; E, plasma eluate, platelets, CaCl_2 , AHF and PACG.

† Kindly supplied by Biologics Control Laboratory, National Institutes of Health, Bethesda, Md.

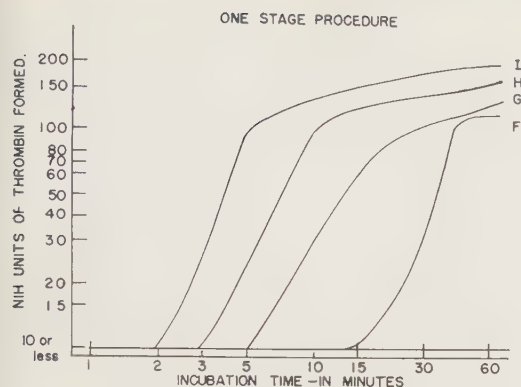


FIG. 2. Formation of thrombin in plasma eluate, serum eluate, platelets and CaCl_2 in the presence of PAcG, AHF and PAcG plus AHF. F, plasma eluate, serum eluate, platelets and CaCl_2 ; G, plasma eluate, serum eluate, platelets, CaCl_2 and PAcG; H, plasma eluate, serum eluate, platelets, CaCl_2 and AHF; I, plasma eluate, serum eluate, platelets, CaCl_2 , AHF and PAcG.

this procedure serum eluate, platelets and CaCl_2 with and without AHF or PAcG were incubated for 20 minutes at 26°C and the plasma eluate was then added to the incubation mixture. The volumes for the different reagents in this procedure were the same as in the one-stage thrombin activation procedure. The prothrombin converting activity of the incubation mixture was assessed by recording the ability and the velocity of the reaction mixture to form thrombin upon addition of a plasma eluate and incubation at 26°C . The rate of thrombin formation was followed in the same manner as in the one-stage thrombin formation procedure. Details of representative individual experiments are included in Fig. 1, 2 and 3.

Results. In any interpretation of the various reaction rates of the different factors controlling the formation of thrombin, consideration must be given to the law of mass action. Cognizance must also be taken of the possible presence of other clotting factors or inhibitors as impurities in the preparations employed, however small these contaminations may be. Nevertheless, it is felt that only quantitative and not qualitative differences may occur when corresponding reactions are executed with more purified preparations. Data presented in the various figures constitute mean values of 3 or 4 identical experi-

ments. The clotting times did not differ by more than 10% for each group of identical experiments.

In the curves of Fig. 1 and 2, depicting simultaneous mixing of the reactants, the rate of thrombin formation represents the summation of all intermediate interactions involved in the elaboration of thrombin. As can be seen from curve A of Fig. 1, incubation of the bovine plasma eluate with platelets and CaCl_2 yielded only traces of thrombin. However, in the presence of either serum eluate (curve B of Fig. 1), or AHF (curve C of Fig. 1), or PAcG (curve D of Fig. 1), prothrombin conversion became more pronounced. As can be seen from Fig. 1 marked thrombin formation did not commence until after 10 to 15 minutes of incubation. Once thrombin formation began, the rate of conversion of prothrombin to thrombin increased rapidly. In the presence of both AHF and PAcG (curve E of Fig. 1), thrombin evolution was relatively fast: 80 NIH units were elaborated after 20 minutes compared with 29 units with PAcG, 20 NIH units with AHF and 15 NIH units with serum eluate. Addition of either serum eluate, or of PAcG, or of AHF, or of AHF and PAcG seemed to influence mainly the rate but not the amount of thrombin formation.

The data in Fig. 2 indicate that addition of

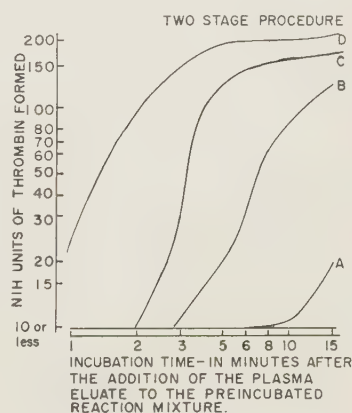


FIG. 3. Formation of prothrombin converting activity on 20 min. incubation of serum eluate, platelets and CaCl_2 in the presence of PAcG, AHF and PAcG plus AHF. A, serum eluate, platelets and CaCl_2 ; B, serum eluate, platelets, CaCl_2 and PAcG; C, serum eluate, platelets, CaCl_2 and AHF; D, serum eluate, platelets, CaCl_2 , PAcG and AHF.

serum eluate to the plasma eluate enhanced the accelerating influence of either PAcG or AHF in the utilization of prothrombin. In the presence of AHF (curve H of Fig. 2) there was both an increase in the yield and the rate of thrombin formation after 10 minutes of incubation (112 NIH units) compared with PAcG (30 NIH units, curve G of Fig. 2). These observations substantiate the concept of Brinkhous and his associates(4) that AHF augments the utilization of prothrombin. In the presence of both AHF and PAcG (curve I of Fig. 2), the lag period of prothrombin conversion was further shortened; about 26 NIH thrombin units were formed within 3 minutes, while only traces of thrombin were elaborated during the same period of incubation with either AHF or PAcG alone. Comparison of the data of Fig. 1 and 2 indicate that AHF as well as PAcG in the presence of serum eluate caused a more pronounced shortening in the prothrombin utilization than in the absence of serum eluate. The quantity of thrombin formed in a mixture of plasma eluate, platelets and calcium at any given time not only was dependent on the amount of prothrombin present but also on the rate of its conversion controlled by the presence and quantity of such factors as AHF, PAcG and those present in serum eluate. Replacing either platelets or CaCl_2 with veronal buffer in the experiments did not yield any measurable thrombin formation.

In an attempt to reduce further the lag period observed in the one-stage thrombin formation procedure, those factors which apparently participate in the conversion of prothrombin to thrombin were preincubated for 20 minutes at 26°C before addition of the plasma eluate. The results illustrated in Fig. 2 and 3 show that preincubation of serum eluate, platelets and CaCl_2 in the presence of PAcG or AHF or both PAcG and AHF before addition of the plasma eluate to the incubation mixture, resulted in a marked enhancement in the prothrombin conversion compared with the one-stage thrombin formation procedure. It appears from these data that AHF as well as PAcG interact with factor(s) in serum eluate in the presence of platelets

and calcium prior to the activation of prothrombin. In the presence of AHF (curve C of Fig. 3) a more potent prothrombin activator seemed to evolve than in the presence of PAcG (curve B of Fig. 3). This is in further agreement with the finding of Brinkhous and his associates(4) that AHF greatly enhances the utilization of prothrombin. Jensen, Gray and Schaefer(10) have presented evidence that PAcG accelerates the interaction of platelets and factor(s) in a serum eluate in the presence of CaCl_2 yielding a prothrombin conversion factor. With both AHF and PAcG in the preincubation reaction mixture, (curve D of Fig. 3), 24 NIH thrombin units were already formed within 1 minute after addition of the plasma eluate. In the presence of AHF only (curve C of Fig. 3) and PAcG only (curve B of Fig. 3) 2.5 minutes and 5 minutes respectively were required to yield the same amount of thrombin. Replacement of platelets or of CaCl_2 with veronal buffer did not yield any prothrombin converting activity. Serum eluate, AHF, PAcG, platelets and CaCl_2 after incubation for 20 minutes at 26°C produced a clot with fibrinogen in 9 minutes, indicating the presence of only traces of prothrombin in the above preparations.

A comparison of the rates of thrombin formation (Fig. 2 and 3) shows that preincubation of AHF or PAcG or both with serum eluate, platelets and CaCl_2 greatly reduced the lag period in the conversion of prothrombin to thrombin. It appears from the data in Fig. 2 and 3 that regardless of whether the prothrombin converting complex was formed in one stage or two, approximately the same amount of thrombin was formed. The main difference between the one and 2-stage reaction was in the rate of prothrombin conversion. The greater velocity of thrombin formation in the 2-stage procedure indicates that during preincubation precursory interactions of certain factors had been initiated or completed.

Preincubation of either platelets, AHF and CaCl_2 or of platelets, PAcG and CaCl_2 or of platelets, AHF, PAcG and CaCl_2 before addition of either plasma eluate or of plasma

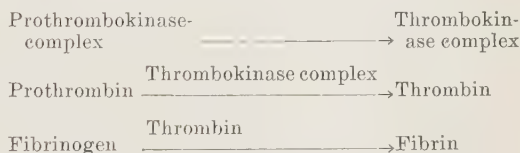
eluate plus serum eluate to the preincubation mixture resulted in approximately the same rates of thrombin elaboration as was found in the one-stage thrombin procedure (Fig. 1 and 2). These findings indicate that no interaction between the various factors had taken place during the preincubation period under these conditions. It appears from these observations that AHF as well as PAcG will interact with platelets and calcium ions only in the presence of factor(s) in serum or plasma eluate yielding prothrombin converting activity.

Discussion. The data presented point to the existence of several factors in blood which react, perhaps in stages, to produce a powerful activator of prothrombin, called the thrombokinase-complex.[‡] Factors concerned in the thrombokinase forming system are AHF, PAcG, factor(s) in serum or plasma eluate, platelets and calcium ions. Apparently PAcG and AHF will react with platelets and calcium only in the presence of factor(s) in serum or plasma eluate yielding prothrombin converting activity. Whether the activities of the thrombokinase generated with and without AHF or PAcG differ qualitatively as well as quantitatively remains to be determined. Applying these results to the mechanism of fibrin formation in blood, the role of the different natural anticoagulants must be taken into account.

The findings reported are in agreement with the concept of Biggs(12) that the Christmas factor* (present in the eluate of plasma or serum), AHF, factor V* (PAcG), factor VII* (present in the eluate of plasma or serum), platelets and calcium ions are all required for the generation of a "complete" thromboplastin[§] (thrombokinase). According to Spurling and King(13), plasma thromboplastin component^{||} (present in plasma or

serum eluate), AHF, platelets, rough surfaces and calcium are required for maximum thromboplastin (thrombokinase) production *in vitro*. These investigators, apparently, did not include PAcG in their concept of thrombokinase formation. Flynn and Coon(14) have presented evidence that brain thromboplastin extract, stable factor* (present in plasma or serum eluate) and labile factor* (PAcG) react together prior to the activation of prothrombin. According to Seegers(15), the plasma platelet cofactor 1* (AHF) reacts with platelet factor 3* to form threone which in combination with serum accelerator globulin* (derived from PAcG) will convert prothrombin to thrombin. Mann and Hurn (16) observed that platelets interact with a plasma—or serum factor (cothromboplastin)* before interaction with prothrombin takes place.

The data presented here as well as the observations of others already cited are in harmony with the 3 stage concept of fibrin formation as formulated by Milstone(11).



The prothrombokinase complex would include such factors as platelets, AHF, PAcG, factor(s) in serum or plasma eluate and calcium. For the thrombokinase complex formation, platelets, factor(s) in serum or plasma eluate and calcium seem to be essential, while AHF as well as PAcG will enhance its formation. The fundamental phase of the blood clotting mechanism seems to involve the generation of a thrombokinase. Once sufficient thrombokinase is formed, the conversion of prothrombin to thrombin by thrombokinase and the conversion of fibrinogen to fibrin by thrombin proceed comparatively rapidly. The time required for the thrombokinase-forming reactions determines the blood coagulation time. The nature and sequences in the interaction between the various factors, yielding thrombokinase, have yet to be elucidated. It is known that AHF and PAcG are

[‡] Following the proposal of Milstone(11), thrombokinase is defined as an agent which has the capacity to convert prothrombin to thrombin directly.

[§] Biggs defines thromboplastin as a substance which is directly responsible for conversion of prothrombin to thrombin in the presence of calcium ions.

^{||} Plasma thromboplastin component is assumed to be identical with the Christmas factor(12).

utilized during the clotting of blood. Whether they become a component part of the thrombokinase complex is at present not known. According to Owren and his associates(17) convertin (obtained by the interaction of brain extract and proconvertin, present in serum or plasma eluate) and accelerin[†] combine to form prothrombinase (thrombokinase) which in the presence of calcium produces rapid conversion of prothrombin to thrombin. Whether the calcium necessary for the formation of thrombokinase becomes an integral component of the thrombokinase complex or whether it serves merely as a catalyst is not known.

Summary. Plasma accelerator globulin, antihemophilic factor as well as serum eluate were found to produce increased thrombin elaboration in a reaction mixture consisting of bovine plasma eluate, platelets and calcium ions. In the presence of both plasma accelerator globulin and antihemophilic factor, conversion of prothrombin to thrombin became accelerated. Addition of serum eluate to the plasma eluate enhanced the accelerating influence of either plasma accelerator globulin, of antihemophilic factor or of both in the utilization of prothrombin. Upon preincubation of those factors which are supposed to participate in the conversion of prothrombin, before addition of a plasma eluate to the incubation mixture, it was found that more prothrombin was converted in a shorter period of time than in the one-stage thrombin formation procedure. The greater velocity of thrombin formation in the two-stage pro-

cedure indicated that precursory interactions of certain factors had taken place during the initial incubation. Evidence has been obtained that antihemophilic factor as well as plasma accelerator globulin will interact with platelets and calcium ions only in the presence of factor(s) in serum or plasma eluate yielding prothrombin converting activity.

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[†] Obtained by addition of thrombin to prothrombin and proconvertin free bovine plasma. Accelerin was then precipitated by dilution and acidification. The preparation may have been contaminated with AHF.

Influence of Cortisone on Experimental Viral Infection. II. Effects on Antibody Formation and Acquired Immunity.* (22136)

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It has been demonstrated that infection of a number of mammalian species with a variety of microorganisms is altered to the detriment of the host by cortisone (see reviews (1,2)). It has also been established with less unanimity that the formation of humoral antibody to non-replicating antigens is suppressed by the administration of cortisone (3,4). However, few studies (5,6) have been published of the influence of cortisone on the development of circulating antibody during actual infection. Perhaps the rapidly lethal outcome of most experimental infections studied precludes the formation of detectable antibody unless antimicrobial therapy is used to arrest infection (5,6).

In the present study, 2 avirulent influenza A virus strains capable of multiplication to high titer without the concomitants of pneumonia or death have been used to permit study of antibody formation resulting from unrestricted multiplication of a viable antigen. In a companion experiment, study has been made of the effectiveness of the immunity so acquired in resisting challenge with a virulent strain of one of the viruses used in immunization.

Materials and methods. *Mice.* CFW mice which averaged 20 g in weight were employed in groups of 6-21 as indicated in the protocols. Mice were inoculated intranasally with viral suspensions while under light ether anesthesia. Blood for antibody determinations was removed under chloroform anesthesia by cardiac puncture exsanguination. *Viruses.* The 1947 FM-1 influenza A strain was employed in two forms: 1) as unadapted first mouse lung passage virus (10% mouse lung suspension), and 2) as a 10% mouse lung suspension of 29th

passage mouse-adapted virus. A 1950 non-mouse-adapted influenza A virus (Rockefeller Institute JB strain) (7) was also employed as undiluted allantoic fluid. *Cortisone.* Cortisone was used in the form of cortisone acetate (Cortone® Merck); the aqueous vehicle used in its suspension was employed for the subcutaneous injection of control animals.† *RDE.* The filtrate of a 48-hour neopeptone broth culture which had been inoculated with the Inaba strain of *Vibrio comma* and incubated at 37°C was used as receptor destroying enzyme to remove serum inhibitor. *Viral titrations.* Assay of pulmonary virus was accomplished by inoculation of four 10-day-old White Leghorn embryos with the indicated dilution of mouse lung. Allantoic fluids were harvested after 40-48 hour incubation at 35°C and titered for hemagglutinin at 1:4 dilution with 1% human "O" RBC. MID₅₀ (mouse infective dose) was determined as previously described (7). *Scoring of pulmonary lesions.* When it became apparent that cortisone did not kill control mice under the conditions employed, the conventional maximum score method (8) (which considers mortality) was used in grading pulmonary lesions, but with a percentile expression of results to facilitate comparison among mouse groups of varying size. *Antibody titrations.* Sera were heated at 56°C for 30 minutes prior to their use. Neutralizing antibody was measured by the mouse-egg technic which can determine neutralization in mice of non-mouse-adapted virus (7). Hemagglutination-inhibiting antibody was measured by a method described earlier (9) after prior incubation of sera with equal volumes of RDE for 18 hours. Human "O" RBC in initial concentrations of 1% were used.

Results. *Effect of cortisone on formation of antibody in mice infected with influenza A*

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‡ These preparations were generously supplied by Merck & Co., Rahway, N. J.

TABLE I. Effect of Varying Doses of Cortisone on Formation of Neutralizing Antibody in Mice Infected with Influenza A Virus (JB Strain).

Serum from mice inj. with:		Neutralizing antibody titer v.s.	
Cortisone (mg)	Virus	50 MID ₅₀ *	5000 MID ₅₀
0	+	>32†	12
5	+	>32	12
1	+	>32	6
2.5	+	6	<4
q 3 days × 6			
5	0	<4	<4

* Mouse infectious doses.

† Reciprocal of serum dilution 50% neutralization.

viruses. Formation of neutralizing or hemagglutination-inhibiting antibody was suppressed during infection of cortisone-injected mice with either of 2 strains of influenza A virus. In one experiment groups of 18-21 mice were killed with chloroform and bled from the heart 19 days following the intranasal inoculation of the JB strain of influenza A virus and the administration of cortisone or control material. The results of titrations of serum neutralizing antibody are presented in Table I. Definite suppression of neutralizing antibody formation followed prolonged cortisone dosage initiated on the day of infection, but was not evident after single injections of 1 and 5 mg of cortisone administered at the time of viral inoculation.

In a second experiment, mice were inoculated with unadapted FM-1 (1947) virus, then subjected to either of two cortisone regimens, as shown in Table II. One schedule consisted of administration of 5 mg doses on the day preceding, the day of, and the day after virus inoculation; the second schedule involved the protracted administration of smaller doses as used in the first experiment. Pooled sera (15 mice/group) were obtained on the 18th day and titrated for hemagglutination-inhibiting antibody. Results are shown in Table II. As in the first experiment, only prolonged administration of cortisone inhibited development of antibody.

Effect of cortisone on acquired active immunity against FM-1 virus. Six groups of 6 mice each were inoculated intranasally with avirulent non-adapted FM-1 virus or saline

(Table III): Fourteen days later mice were challenged with mouse-virulent FM-1. Mice received concomitant injections of cortisone as indicated. No fatalities occurred in mice previously infected with FM-1, indicating that such mice possessed at least partial immunity when compared with mice not previously infected with virus. However, pulmonary lesions involving 33% of total lung volume were found in mice given large amounts (15 mg) of cortisone at the time of challenge. It is also of interest that two groups of mice given cortisone (5 or 15 mg) had demonstrable pulmonary virus 7 days after challenge, in contrast to mice injected with saline, who were found to have none. Thus, cortisone in large doses may lower acquired host resistance to allow viral multiplication, and result in the appearance of manifest disease, as well.

Discussion. To the author's knowledge the present study is the only published investigation which describes inhibition by cortisone of antibody formation during infection. A study by Kass *et al.* (10) with a virulent influenza A strain failed to demonstrate humoral antibody in either control or cortisone-treated mice 6 days after inoculation—leaving the question of antibody inhibition unanswered. Studies of streptococcal infection in rabbits (5) and man (6) have shown no suppression of anti-streptolysin O formation with cortisone. Germuth *et al.* (11) found no interference by cortisone with the development of an immune response to pneumococcal infection of the rabbit, but made no study of circulating antibody.

The demonstration in the present study

TABLE II. Effect of Varying Doses of Cortisone on Formation of Hemagglutination-Inhibiting Antibodies in Mice Infected with Influenza A (FM-1) Virus.

Serum from mice inj. with:		Antibody titers†
Cortisone (mg)	Virus*	
0	+	64
5 × 3	+	32
0	0	<8
5 × 3	0	<8
2.5 q 3d × 6	+	<8

* 10⁶ EID₅₀.

† Reciprocal of highest dilution of serum which inhibited hemagglutination with 8 hemagglutinating units of virus.

TABLE III. Effect of Cortisone on Acquired Active Immunity against FM-1 Virus; Challenge of Immunized Mice with Virulent Virus.

Day 1		Day 14		Day 21	
Initial inoculation	Challenge inoculum*	Cortisone (mg)	D/T†	Lesions, maximum score (%)	Virus‡ in lungs
FM-1 (1st mouse pass.)	FM-1 (29th pass.)	5	0/6	10	+
"	"	0	"	0	0
"	"	5 × 3§	"	33	+
Saline	FM-1 (29th pass.)	0	4/6	72	
"	"	5	4/5	80	
FM-1 (1st mouse pass.)	Saline	0	0/6	3	0

* 10^6 MID₅₀. † No. of dead mice/total in group.

‡ +, 10^{-2} lung suspension infects eggs; 0, 10^{-2} lung suspension does not infect eggs.

§ On days 13, 14, 15.

that antibody suppression is dependent on cortisone dosage suggests an explanation for the inhibition of antibody formation noted here but not observed by others. Species difference would not appear to be an important factor in view of evidence of profound inhibition by cortisone in the rabbit of antibody formation to non-multiplying antigens(3,4).

The present study has demonstrated that cortisone may suppress the formation of antibody during the course of a viral infection, and further, that the immunity conferred by infection may be partially surmounted by administration of cortisone with a challenge inoculum. However, reduction in pre-existing acquired immunity is effected by cortisone dosages inadequate to suppress antibody formation. Thus, the persistence of pulmonary virus and the induction of pneumonia in immune mice given cortisone cannot be ascribed to effects on circulating antibody. These findings are in accord with previous observations that 1) lethal infections may be rapidly induced with cortisone in naturally resistant hosts(5,12) and 2) that increased viral multiplication and mortality may be effected by cortisone in a host (the chick embryo) incapable of demonstrable antibody formation(13).

It is conceivable that the demonstrated multiplication or persistence of virus in an immune host may be explicable on the basis of cortisone-induced reactivation of pre-existing pulmonary virus, but thus far the phenomenon of viral reactivation with cortisone has been studied only in the chick embryo(14).

Summary. 1) The formation of neutralizing and hemagglutination-inhibiting antibodies was suppressed by cortisone administered

during infection of mice with influenza A viruses. 2) Suppression of antibody was dependent on prolonged administration of cortisone and was not effected by large doses given at the onset of infection. 3) Acquired active immunity induced by infection of mice with avirulent virus was partially surmounted by administration of cortisone with a challenge inoculum of virulent virus. 4) Cortisone in doses insufficient to suppress antibody formation induced lesions and persistence of pulmonary virus following challenge of mice with previously acquired immunity.

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Plasma Penicillin Levels from Oral Penicillins V and G and Intramuscular Penicillin G.* (22137)

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Penicillin V (phenoxymethyl penicillin), because of its stability at low pH ranges, its low solubility in an acid medium and rapid conversion to the water soluble salt at neutral or alkaline pH, has been suggested as possibly superior to penicillin G for oral administration(1-3). The available information concerning penicillin V was reviewed recently by Jones and Finland(4) who, in a controlled study, compared the penicillin activity in the plasma of normal subjects following ingestion of single tablets of 200,000 units of penicillins G and V. They found that penicillin V produced higher and more prolonged blood levels than did penicillin G as judged by the anti-streptococcal and antistaphylococcal activity of the plasma, particularly when the doses were taken after a meal. They also showed that the numerical value assigned to the concentration of penicillin in plasma depends on the strain used in the assay and on the type of penicillin (*i.e.*, V or G) in the standard solution employed as the control from which the concentrations are computed. Wright *et al.*(5) also showed that differences found by microbiological assay methods depend mainly on the assay organism but also on the medium used for the dilutions. They too found considerably higher blood concentrations from penicillin V than from the same 200,000 unit dose of penicillin G given orally, and, in addition, they showed that twice as much of penicillin V is excreted as the active form in the urine.

The present paper presents further comparisons of levels of penicillin in plasma resulting from the same amounts of penicillins

V and G given orally at various dosage levels, and these, in turn, are compared with levels obtained from intramuscular injection of the same amount of penicillin G.

Materials and methods. The subjects were 8 normal young men and 24 ward patients, 3 of whom were women; their ages ranged from 15 to 86 (avg 50 yr) and their weights ranged from 40 to 95 kg (avg 66 kg). None had recently received any antimicrobial agents. All had grossly normal renal function as judged by routine clinical laboratory tests except one who had renal insufficiency with nonprotein nitrogen levels ranging as high as 145 mg%. All were able to and did ingest normal quantities of food and liquids, but the intakes were not controlled. Three dosage forms of penicillin were used, *viz.* (1) the free acid of penicillin V in 200000 unit tablets, (2) buffered potassium penicillin G ("Pentids," Squibb) in 200000 unit tablets, and (3) aqueous potassium penicillin G intramuscularly. These were given at 3 dosage levels, *viz.* 200000 units, 400000 units and 1000000 units; individual doses were separated by at least 48 hours. Every dose was given $\frac{1}{2}$ hr after breakfast. All subjects received both penicillin V and G orally at least at one and the same dosage level and many also received an i.m. injection of the same number of units of penicillin G. Three of the young men received all 3 dosage forms at all 3 levels, and 5 subjects, including these 3, received all 3 dosage forms at each of the 3 levels. Assays for penicillin in plasma were done by the 2-fold dilution method in broth as in the previous study(4) except that only *Strep. 98* was used as the assay organism and the numerical values assigned were based only on comparisons with a standard of penicillin G. As in the previous study(4), graphic comparisons will be based only on the maxi-

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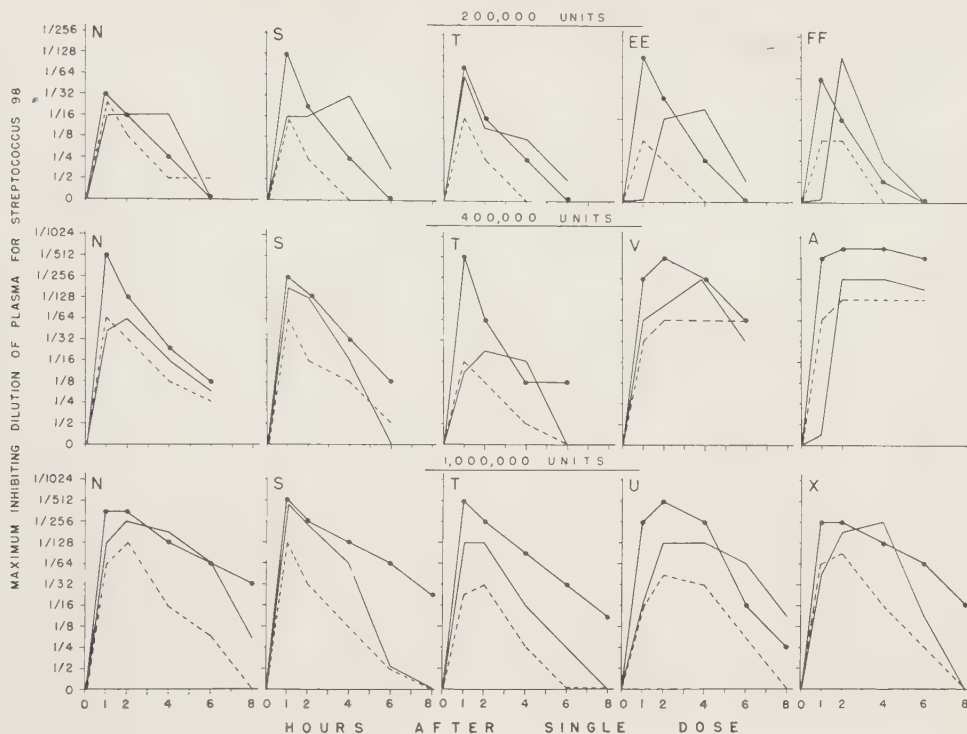


FIG. 1. Plasma penicillin levels in same subjects after same dose of penicillin given as: Penicillin V orally (—), buffered penicillin G orally (---), and penicillin G intramuscularly (•—•).

imum dilutions of plasma which inhibit *Strep. 98*; in these studies the standard solution of 1 $\mu\text{g}/\text{ml}$ of penicillin G inhibited the test strain at a dilution of 1:128, and 1 μg of the solution of penicillin V inhibited at 1:64. Blood for the assays was obtained before each dose and 1, 2, 4 and 6 hours after the dose in every instance, and also 8 hours after the 1 million unit dose. All specimens of plasma taken immediately prior to a dose showed no detectable penicillin activity.

Results. Comparative Levels in the Same Subject. The levels of antistreptococcal activity obtained in the plasmas of 5 subjects who received all 3 dosage forms at the same dosage level are shown graphically in Fig. 1. The letters in each panel identify the subjects. It is seen from this figure the relative levels of penicillin activity in plasma obtained from the 3 dosage forms varied with the dose. In general, i. m. penicillin G produced higher peak levels more rapidly than the other forms. With the dose of 200000 units these were not

as well sustained as from oral penicillin V, whereas with the higher doses they were bet-

TABLE I. Average Concentrations of Penicillin in the Same 5 Subjects after the Same Dose of Penicillin Given in 3 Forms.

Dose units* ($\times 1000$)	Hr after dose	Dosage form—		
		Penicillin G, orally	Penicillin V, orally	Penicillin G, intramusc.
Avg level† in plasma, $\mu\text{g}/\text{ml}$				
200	1	.22	.21	1.63
	2	.12	.35	.39
	4	.018	.19	.06
	6	.005	.046	0
400	1	.99	.47	7.5
	2	.93	1.8	3.97
	4	.40	1.05	1.40
	6	.20	.22	.70
1000	1	1.01	1.16	7.0
	2	1.4	2.69	6.07
	4	.39	1.40	2.53
	6	.11	.29	.82
	8	.05	.08	.26

* 200000 units = approximately 120 mg penicillin, either as G or V.

† Determined by broth-dilution method using *Streptococcus 98*, and comparing with a penicillin G standard solution.

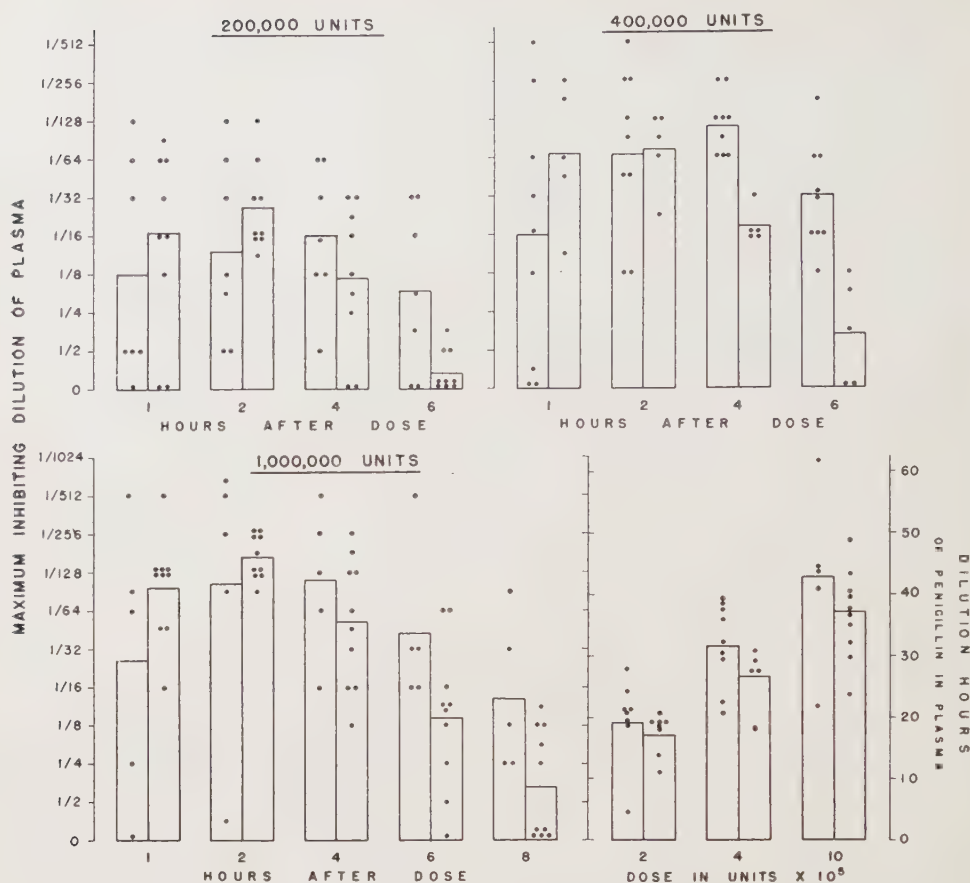


FIG. 2. Effect of age on levels of penicillin in plasma after oral penicillin V. Subjects 60 yr old and over (left) compared with those under 60 (right).

ter sustained and at higher levels. Calculations of the areas ("dilution-hours") under the curves following the 200000 unit doses showed no significant difference between oral penicillin V and i.m. penicillin G ($P = >0.1$), but the differences between both of these and the values for oral penicillin G were significant ($P = <0.02$ in each instance). After the 400000 unit doses, the levels were more or less uniformly higher with i. m. penicillin G than after oral penicillin V, and these in turn were higher than those following oral penicillin G. Calculations of the areas under these curves, however, yield values that are not statistically significant; this is due, in large part, to the fact that plasma-penicillin levels were not obtained after 6 hours and these are not readily extrapolated. On the other hand, 1 million units of i. m. penicillin

G gave the highest levels of penicillin activity throughout, and oral penicillin V yielded higher levels than oral penicillin G. In this instance, the differences between the areas under the curves are statistically significant in every instance.

The average plasma penicillin levels, calculated as penicillin G, are shown in Table I; they show the same differences that are reflected in Fig. 1.

Effect of Age. Since a number of the subjects were in the older age group, an attempt was made to determine the effect of this factor on the levels of penicillin in the blood. This was done only for oral penicillin V. The data are charted in Fig. 2. For all 3 dosage levels, the penicillin activity in the plasma of the subjects over 60 years old rose more slowly, achieved a peak later, and thereafter

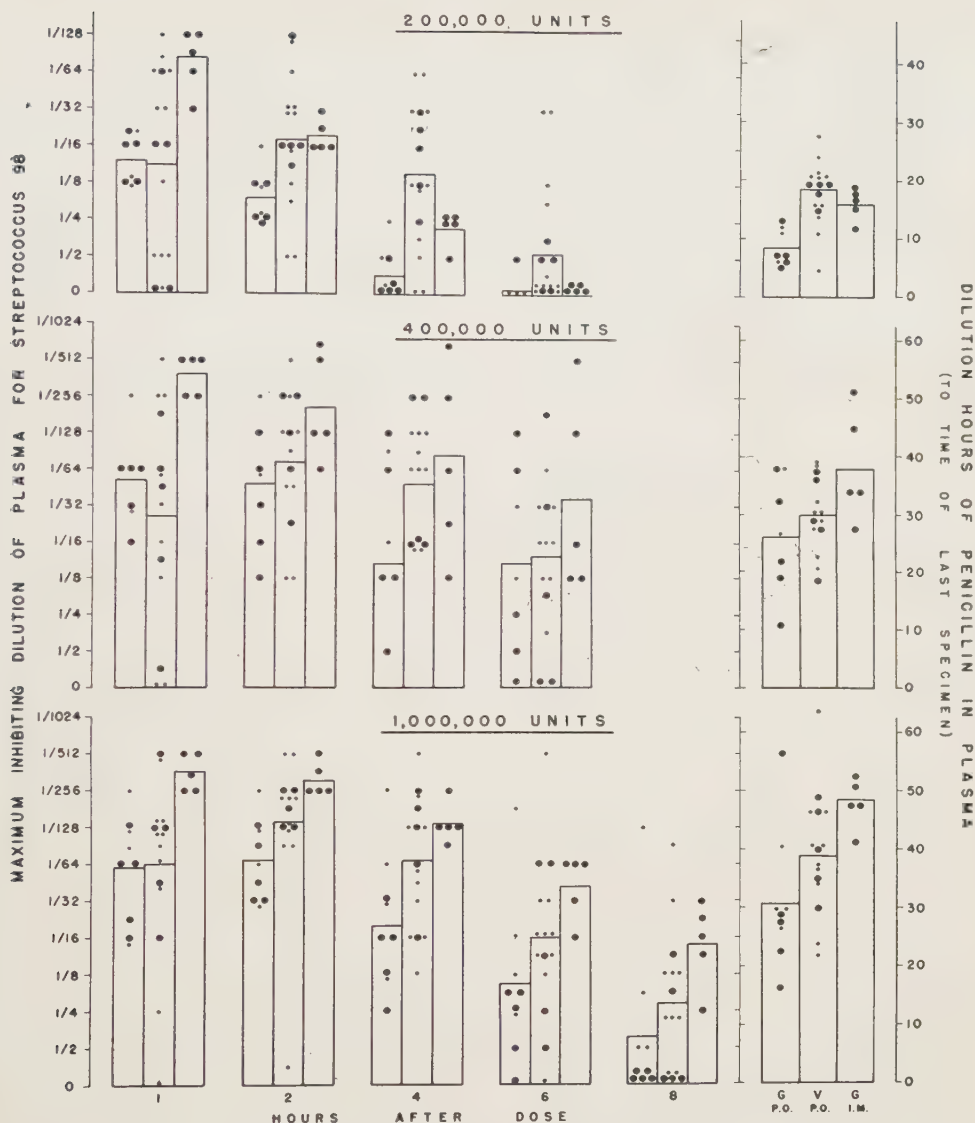


FIG. 3. Penicillin levels in plasma following administration of penicillin V orally (center) compared with penicillin G, orally (left) and intramuscularly (right). The large dots represent subjects who received all 3 dosage forms at the stated intervals.

were sustained better than in the younger ones. This may be a reflection of differences in renal function. This figure also shows the areas (dilution-hours) under the curves of the plasma penicillin levels; these values and their means were higher in the older subjects at each of the 3 dosage levels.

Further Comparisons of the 3 Dosage Forms. The levels of penicillin in plasma obtained in all the subjects studied and the

mean values for each dosage form at each dose level are charted in Fig. 3. In this figure the large dots represent the values in the subjects for whom data are available for all 3 forms at the stated dose levels. At the right are also shown the areas or "dilution-hours" under the curves of the plasma penicillin levels and the means of these values. Here again it is seen that penicillin V is superior to penicillin G at each dose level. The dose of

200000 units of i. m. penicillin G gave higher and earlier peak levels but these were less sustained than from oral penicillin V; the average number of dilution-hours, however, was quite similar for these 2 forms at this level, and these were both greater than from oral penicillin G. After doses of 400000 units and 1000000 units, on the other hand, i. m. penicillin G gave the highest and best sustained levels and oral penicillin V gave higher and better sustained levels than oral penicillin G; these differences are reflected in the number of dilution hours of penicillin in the plasma.

Summary. Oral penicillin V gave higher and better sustained levels of penicillin activity in the plasma than oral buffered potassium penicillin G at each of 3 dosage levels, *viz.* 200000, 400000 and 1 million units. Intramuscular penicillin G yielded higher and better sustained levels than oral penicillin V in equivalent doses given at levels of 400000 or 1 million units. An i. m. dose of 200000

units of penicillin G produced higher peak levels and these occurred earlier but they were less well sustained than with this amount of oral penicillin V; the total amount of penicillin absorbed from this amount of penicillin was not significantly different for these 2 dosage forms. Persons over 60 years of age attained peak levels later; in general they had higher and better sustained levels of penicillin in the plasma from any given dose than did younger individuals.

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Effect of Dietary Protein and Energy on Chick Liver Fat Accumulation.*† (22138)

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Best and Huntsman(1) discovered that a dietary deficiency of choline resulted in an excessive accumulation of fat in rat livers. Later work, Harper *et al.*(2) indicated that such compounds as glycine, serine, methionine and betaine, which may function as choline precursors, are able to reduce the level of liver fat in rats on choline-free diets. Channon and Wilkinson(3) and Best and Huntsman(4) suggested that the deposition of liver fat might

TABLE I. Basal Diet for Preliminary Period.

Ingredient	%
Glucose	54.4
Soy protein*	31.6
Soybean oil	4.0
Cellulose	3.0
Methionine	.5
Mineral mix†	5.5
Vit.-antibiotic mix‡	1.0
Total	100.0

* Drackett Assay Protein C-1.

† Provides per lb diet: NaCl 0.5%, Ca 1.6%, P 0.88%, K 1.3 g, Zn 1.08 mg, Mn 33.3 mg, Fe 30.6 mg, Co 0.6 mg, Cu 1.2 mg, Mg 300 mg, and I 5.0 mg.

‡ Provides per lb diet: Vit. A 4000 I.U., Vit. D₃ 500 I.C.U., alpha-tocopherol 7.0 mg, menadione 0.6 mg, choline Cl 868 mg, inositol 455 mg, niacin 45 mg, calcium pantothenate 10 mg, riboflavin 3.6 mg, pyridoxine-HCl 2.7 mg, thianine-HCl 1.8 mg, folic acid 1.4 mg, p-amino benzoic acid 0.9 mg, biotin 90 µg, vit. B₁₂ 6 µg, penicillin 4 mg.

* Part of thesis by senior author in partial fulfillment of requirements for degree Doctor of Philosophy, Iowa State College.

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TABLE II. Experimental Diets.

* Ingredient	Calculated dietary protein level									
	14	18	22	26	30	14	18	22	26	30
	Low energy*					High energy†				
Glucose	52.3	47.6	42.9	38.3	33.5	66.6	61.6	56.4	51.4	46.2
Soy protein	16.4	21.2	26.1	30.9	35.8	16.4	21.2	26.1	30.9	35.8
Soybean oil	1.0	1.0	1.0	1.0	1.0	10.0	10.2	10.5	10.7	11.0
Cellulose	23.3	23.2	23.0	22.8	22.7	—	—	—	—	—
Methionine	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5
Mineral mix‡	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Vit.-antibiotic mix§	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

* Calculated to contain 720 C/lb productive energy.

† " " " 1055 C/lb

‡ Minerals supplied as in Table I.

§ Vitamins and antibiotic supplied as in Table I.

be linked to protein metabolism. In this respect Eckstein(5) found that only methionine exhibited a lipotropic effect when each of the 10 essential amino acids was incorporated singly into a choline-free diet. Threonine(6) has been shown to exhibit a lipotropic effect when incorporated into a casein diet complete in all known nutrients, but this phenomenon may have occurred because of the low biological availability of threonine in casein(7). The data of Winje *et al.*(8) support this conception. Recently Lucas and Ridout(9) substantiated the suggestion of Harper *et al.*(2) that the level of protein in the diet has an influence on the accumulation of fat in the livers of rats. They reported a larger percentage of fat in the livers of rats on methionine-supplemented low-protein diets than in the livers of rats on diets higher in protein. As far as the authors are aware, no such effect on liver fat has previously been reported for the chick.

The purpose of this investigation was to study the effect of dietary protein and energy levels on the accumulation of fat in the livers of chicks.

Methods and materials. Eighty day-old chicks were maintained on a nutritionally complete basal diet (Table I) for 10 days after which they were randomly assigned to 40 pens containing 2 birds per pen. At this time 4 pens were allotted to each of the 10 experimental diets (Table II) under a restricted randomized block design. The experimental feed, except that which was in the feeders, was kept refrigerated at 38°F during the experimental period. The birds were

maintained in thermostatically controlled batteries with feed and water supplied *ad libitum* during the experimental period. After 10 days on the experimental diet the birds were sacrificed and their livers were removed and frozen at -20°F. The livers from the birds in each pen were pooled, washed of adhering blood and fatty tissue and blended in a Waring Blendor. The resulting homogenous mixture was dried for 16 hours at 212°F and then ground for analysis. Crude fat determinations were made according to the A.O.A.C.(10) method.

Results. The data in Table III indicate that the energy level of the diet apparently had no marked effect on the chick liver fat. A statistical analysis of the data indicates a significant linear response and a significant quadratic response in percent liver fat due to dietary protein level changes on the high energy diets and the low energy diets, respectively. As shown in Fig. 1, this suggests that the amount of fat in the liver is inversely related to the protein level of the diet.

Discussion. Chicks consuming a high fat (10%) diet accumulated no more fat in their

TABLE III. Effect of Energy Level of Diet on Response to Protein Level Increments.

Level of protein in diet (%)	Liver fat (%)	
	High energy diet (1055 cal/lb)	Low energy diet (720 cal/lb)
14	13.35	12.35
18	13.76	13.58
22	11.25	13.24
26	10.57	11.79
30	10.20	10.54

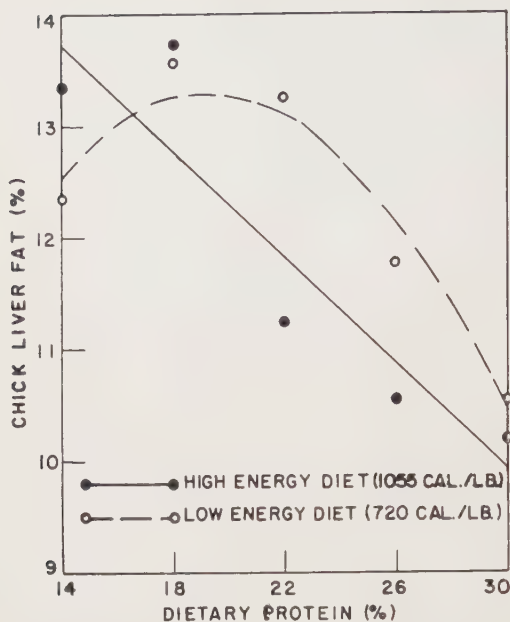


FIG. 1. Effect of high and low energy diets on liver fat content with relation to protein level increments.

livers than those consuming a low fat (1%) diet. This indicates that dietary fat *per se* was not deposited in the chick's liver to any great extent. Harper *et al.*(2) reported a similar effect in rats.

The present work reveals that over a specific (14 to 30%) range there is an inverse relationship between the level of protein in the diet and the amount of fat deposited in the chick's liver. These results are in agreement with those of Lucas and Ridout(9) with rats. The diets in the present study were sufficient in all nutrients known to be required

by the chicks, and these, as well as the energy content of the diet, were held approximately constant over the range of dietary protein levels tested. In most of the previous work using the rat, these extraneous variables had not been as closely controlled.

It is apparent from these data that protein exerts a lipotropic effect not directly involving its choline or choline precursor content.

Summary. The present work reveals that over a range of protein levels from 14 to 30% there is an inverse relationship between the level of protein in the diet and the amount of fat deposited in the chick's liver. The dietary energy level does not appear to influence the chick liver fat level.

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Serum Sodium Concentration of Hypertensive Rats: Relation to NaCl Intake, Blood Pressure and Age.* (22139)

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Alterations of sodium metabolism have been studied intensively in both experimental and clinical hypertension. Among the deviations from normal are changes in total body sodium (1), extracellular fluid volume(2), sodium and water contents of aortic walls(3), as well as plasma and serum sodium concentration. With respect to the last, results have ranged from no apparent change in a very small number of experimentally hypertensive rats(1) to slight but definite increases in larger numbers of hypertensive rats(4) and of hypertensive patients(5). In addition, a relative aversion to NaCl, together with polydipsia and polyuria, has been observed in rats made hypertensive by bilateral renal encapsulation with latex envelopes(6-8). The mechanisms by which this relative NaCl aversion is associated with the induced perinephritis and experimental hypertension are still unclear. An elevated serum Na concentration in hypertensive rats might conceivably be one factor in this relative aversion.

With this possibility in view, the studies now reported were done to determine (a) whether serum Na concentration is increased in rats with established hypertension, (b) whether differences in serum Na concentration can be related to known differences in voluntary NaCl intake and/or to systolic blood pressure and (c) whether age at encapsulation affects serum Na concentration. It has been shown previously that age at encapsulation is one of the factors affecting NaCl aversion(8).

Methods. Male rats of the Sprague-Dawley strain, and of similar ages, were kept in individual cages in a thermoregulated room maintained at $25 \pm 1^\circ\text{C}$, illuminated from

8 a.m. to 6 p.m. They were made hypertensive by enclosing both kidneys in latex rubber capsules(9). A group of 31 normal rats (series I) served as controls. In 23 rats (series II) renal encapsulations were done when they were 50 to 90 days old; in 14 rats (series III) when they were 126 to 165 days old. At least 3 months were allowed for development of hypertension(8). All rats were sacrificed when approximately 300 days old. Prior to sacrifice, *ad libitum* intakes of tap water, .15 M NaCl solution and Purina laboratory chow were measured in 27 rats for 5 days as described earlier(8). Blood pressure was measured by microphonic manometer(10) but without anesthesia(7). At the end of the 5th day the rats were lightly anesthetized by ether inhalation and their necks were carefully shaved. On the 7th day groups of normal and hypertensive rats were sacrificed by guillotine decapitation without anesthesia and 5 to 6 ml of blood were collected directly in test tubes by free flow from the neck vessels without anticoagulant. During collection, the shaved skin of the neck was retracted to prevent contamination of the collected blood by Na from skin or hair. The blood was centrifuged, the serum separated and analyzed for Na by a lithium internal standard Baird flame photometer model number DB2. Sera were diluted 1:100 (using pipettes whose delivery volume was individually checked) in order to provide concentrations within the range of maximum accuracy of the photometer (1.0 to 1.5 mEq./L). Dilutions were made with Na-free distilled water in 100 ml volumetric flasks at 22°C . Each serum sample was bracketed by at least 2 standard solutions, with one Na concentration above and one below the unknown. To determine the precision of the photometer and analytic procedure in general, several aqueous test solutions of NaCl were analyzed. Two were prepared gravimetrically from desiccated NaCl by one of us and analyzed as unknowns by another. Two com-

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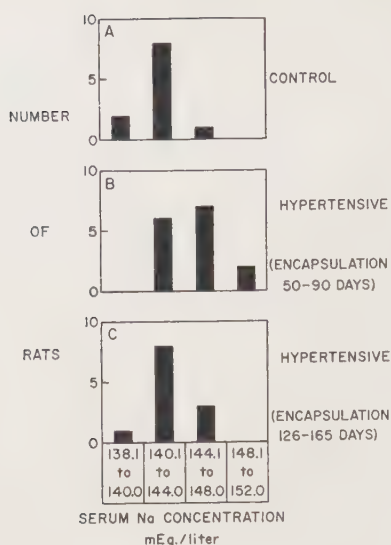


FIG. 1. Frequency distribution of serum Na concentrations (mEq./L) of control and hypertensive rats.

mercially prepared standard solutions[†] of highest accuracy were also analyzed. Typical of these tests are 3 analyses of standards containing 150.3, 100.0 and 5.0 mEq. Na/L which were diluted for analysis 1:100, 1:100, and 1:5 respectively to be in the range of the diluted serum Na. These analyses yielded, in duplicate determinations, final figures of 149.6 and 149.9, 100.0 and 100.0, 5.0 and 5.0 mEq./L respectively. Hence, if each unknown was bracketed by 2 standards, read at least twice and calculated from a separate calibration curve, the precision of the photometer in the range of serum Na concentration was ± 0.5 mEq./L. This precision agrees with that reported by others(5). Fisher's test for significance of differences between means was used for statistical comparison of data from any 2 groups(11).

Results. Fig. 1 shows the range and distribution of serum Na concentrations observed in (A) control rats (B) hypertensive rats following encapsulation at 50 to 90 days

of age and (C) hypertensive rats following encapsulation at 126 to 165 days of age. Serum Na concentrations tended to be slightly higher than normal in those rats that were made hypertensive by encapsulation while still relatively young (Fig. 1B). As shown in Table I this difference, in terms of mean serum Na concentration, was 2.1 mEq./L. and was statistically significant ($P < .01$). On the other hand, rats made hypertensive by renal encapsulation at 126 to 165 days of age had a mean serum Na concentration which was within the normal range (Fig. 1C and Table I).

The mean *ad libitum* intake of NaCl in the form of .15 M NaCl solution by normal rats in this study was 8.2 ± 1.9 ml/100 g B.W./day. For hypertensive rats encapsulated at 50 to 90 days of age the corresponding figure was 3.0 ± 0.8 ml/100 g B.W./day, showing a highly significant reduction ($P < .01$) in agreement with a number of earlier comparisons(6-8). It is tempting to consider the lower NaCl intake simply a direct effect of the higher serum Na concentration but such an inference is not justifiable. Figure 2 shows the individual intakes of .15 M NaCl solution plotted against serum Na concentrations of the corresponding rats (closed circles for controls, crosses for hypertensive rats). The linear correlation in this plot based on individual rats is not good ($r = -.45$, $P < .05$). Hyperbolic, parabolic, logarithmic and exponential functions did not fit the data any better than the simple linear regression equation. Thus, as a group, rats encapsulated at 50 to 90 days of age presented a significant NaCl aversion together with a slight but significant increase in serum Na concentration. Yet in these same rats, considered singly, no close relationship existed between individual serum Na concentrations and their individual NaCl intakes (Fig. 2).

Mean systolic arterial blood pressures in the 3 groups were 138 (control, Series I), 188 (Series II) and 206 (Series III) mm. Hg respectively. The last two values were significantly higher than the control value ($P < .01$), but did not differ significantly

[†] We are grateful to Mr. Sage and Mr. Lightfoot of Baird Associates, Cambridge, for a sample of their reference standard solution. The second solution was a standard for flame photometry and spectrophotometry prepared by Hartmann Leddon Co., Philadelphia, Pa.

TABLE I. Effect of Age at Encapsulation on Serum Na Concentration and Blood Pressure of Normal and Hypertensive Rats.

	No. of series	Mean age at encapsulation (days)	Mean post-operative age (days)	Mean age at sacrifice (days)	No. of rats	Serum Na conc. (mEq./L) *	P	Systolic blood pres. (mm Hg)	P
Control	I	—	—	350 (268-442)†	31	143.0 ± .4†		138 ± 2	
Hypertensive	II	63 (50-90)	246 (160-330)	309 (280-380)	23	145.1 ± .6	<.01	188 ± 7	<.01
	III	150 (126-165)	140 (104-176)	290 (268-345)	14	142.6 ± .7	<.01	206 ± 11	.20

* Each datum can be measured with an accuracy of $\pm .5$ mEq./liter but can be read from a calibration curve with a precision of $\pm .1$ mEq./liter. For purposes of statistical computation the data are tabulated here with 1 more figure than is significant, and the final figure for the mean should be rounded off.

† $\pm$ stand. error of the mean.

‡ Range.

from each other ($P = .20$) and by this criterion the rats of series II and III were equally hypertensive. Yet serum Na concentrations were elevated in one group and normal in the other (Fig. 1, Table I). In both groups plots of individual serum Na concentrations and individual blood pressures (not shown) revealed no close correlation.

Since all groups of rats were sacrificed at approximately the same age, it is evident that those whose kidneys were encapsulated when young were hypertensive somewhat longer than those whose kidneys were encapsulated

later in life. Inasmuch as the young group was the one with elevated mean serum Na concentration, a possible relation between duration of hypertension and serum Na concentration was sought (Fig. 3). Earlier observations have shown that blood pressure is maximally elevated in both old and young rats within 70 days after bilateral latex encapsulation of the kidneys and that the earliest rise is detectable within 14 days(8). Therefore, it was possible to express approximately the duration of hypertension by the number of days between the time of operation and the time of sacrifice for both hypertensive groups (Fig. 3). No close correlation could, however, be established between serum Na concentration and the duration of hypertension.

Discussion. The slight, but significant, elevation of serum Na concentration in rats made hypertensive when young parallels the findings of Holley *et al.*(5) in human essential hypertension and also those of Laramore and Grollman(4) in experimental hypertension in rats with figure of eight ligatures around the kidneys. In the 2 earlier studies, however, age at onset of hypertension and duration of hypertension were not considered with reference to serum Na concentration.

In the present observations, rats whose kidneys were encapsulated when young (50 to 90 days) had a slightly higher mean serum Na concentration while those whose kidneys were encapsulated when older (126 to 165 days) presented a normal distribution of

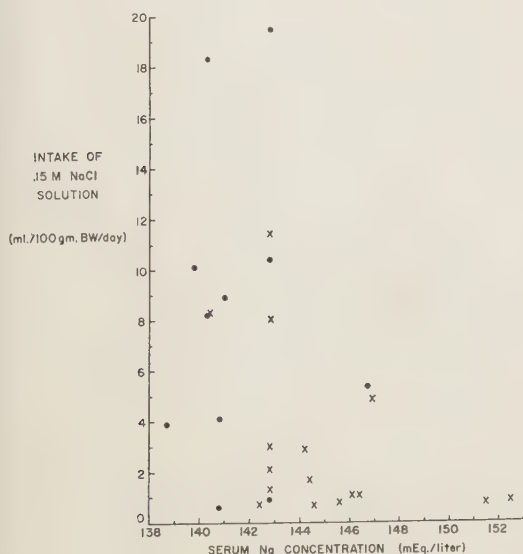


FIG. 2. Individual intakes of .15 M NaCl solution plotted against individual serum Na concentrations of control rats (dot) and rats made hypertensive while young (X).

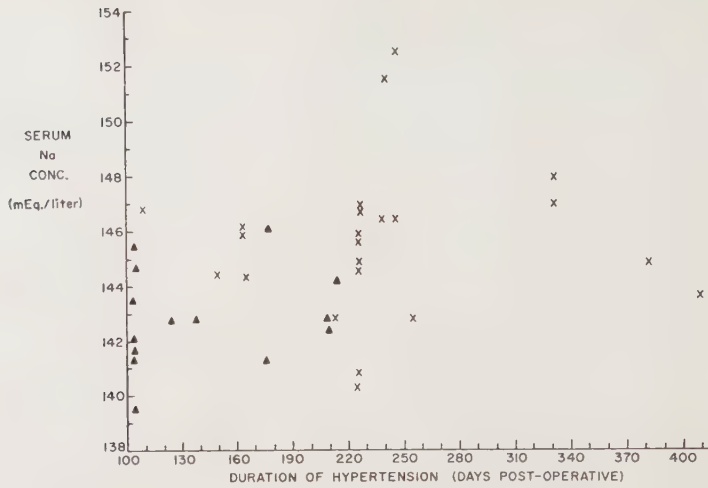


FIG. 3. Serum Na concentration plotted against duration of hypertension for individual rats aged 50 to 90 days (X) and 126 to 165 days (▲) at encapsulation.

serum Na concentrations. A previous study (8) showed that renal encapsulation of younger rats also led to earlier and more definite sodium aversion than did encapsulation later in life. Yet the grade of hypertension was similar with early and late encapsulation. In terms of individual rats, the study reported here has revealed that serum Na concentrations could not be related consistently to the grade of Na aversion, to height of blood pressure, or to duration of hypertension.

It appears, therefore, once more that detailed studies support previous inferences that certain general changes in Na metabolism occur in association with the experimentally hypertensive state under some conditions. Yet a close relationship is not established and further studies are needed to explore possible mechanisms by which the two may conceivably be linked.

Summary. Encapsulating both kidneys of young rats (50 to 90 days) in molded latex envelopes produced hypertension and elevated their mean serum Na concentration slightly (2 mEq./L.) but significantly above that of control rats. The same procedure in older rats (126 to 165 days) produced similar

hypertension but mean serum Na concentration remained within normal limits.

The authors are indebted to Miss Cynthia S. Cook for technical assistance and to Mr. Franklin W. Smith for drawing and photographing the figures.

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Effect of Cholinesterase Inhibitors on Electrolyte Distribution *in vivo*. (22140)

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Mechanisms governing the metabolism of electrolytes at the cellular level remain obscure. It is not known how cells maintain high concentrations of potassium in the face of constant interchange of potassium between intracellular and extracellular compartments. Potassium metabolism may be related to both carbohydrate and acetylcholine metabolism. Observations concerning potassium and acetylcholine have been made *in vitro* on red cells, nerve and muscle. Greig and Holland reported increased permeability of dog erythrocytes due to cholinesterase inhibitors(1). They suggested that cholinesterase activity maintained the selective permeability(2). Other investigators found no direct correlation between inhibition of cholinesterase activity and breakdown of the cellular potassium concentration gradient in the human red cell(3). Recently it has been shown that this erythrocyte synthesizes insufficient acetylcholine to provide energy for accumulation of potassium(4). The relationship between cholinesterase and potassium during neural conduction or muscular contraction would appear to be even more complex. Nachmansohn concluded that acetylcholine increased permeability of nerve membranes (5). Cholinesterase inhibitors increase the rate of entry of sodium into squid axon and decrease that of potassium(6,7). Holland, Dunn and Greig found that acetylcholine caused a loss of potassium from auricular muscle which is reversed by physostigmine (8). They felt that the metabolism of acetylcholine may be correlated with the transfer of sodium and potassium. However, Fenn and co-workers found that acetylcholine caused a small liberation of potassium from muscle, probably due to contraction, and concluded that acetylcholine did not control potassium transfer(9). Little information is

available concerning *in vivo* interrelationships of cholinesterase and potassium. In acute experiments cholinesterase inhibitors in lethal doses cause hyperkalemia(10). However, this occurs in association with anoxemia and acidosis. It was felt that repeated sublethal doses of cholinesterase inhibitors would obviate these alterations and, at the same time, induce chronic disturbance of acetylcholine metabolism. It was not known if the loss of cellular potassium due to cholinesterase inhibitors *in vitro* could be reproduced *in vivo*. If this occurred a diet deficient in potassium would impose an impediment to the replenishment of cellular potassium. The following studies were carried out to determine the effect of cholinesterase inhibitors on tissue electrolytes *in vivo*.

Methods. Male Wistar rats weighing 100 g were given a low potassium diet (0.5 mM potassium per 100 g diet) composed of casein, 24%; glucose, 62%; corn oil, 8%; and salt mixture, 6%(11), plus a daily vitamin supplement of 1/100th of a capsule of Tycopan (Lilly). Distilled water was partaken *ad lib*. Additional rats were given sufficient supplementary potassium (as KCl) to bring the potassium content of their diet to 0.4%; these are referred to as normal potassium rats. Anticholinesterase compounds were administered to potassium deficient and normal potassium rats as follows: Group A1 was composed of control rats injected with 0.5 ml isotonic saline and rats injected with 0.05 mg diisopropylfluorophosphate (DFP) in 0.5 ml isotonic saline; and Group B was composed of potassium deficient controls injected with 0.5 ml propylene glycol and rats on both types of diet injected with 0.15 mg p-nitrophenyl-diethylthionophosphate (Parathion) in 0.5 ml propylene glycol. In these groups subcutaneous injections were given twice weekly starting 27th day of diet. Rats were sacrificed on 44th, 49th and 71st days. An addi-

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tional series of potassium-deficient rats (Group A2) was composed of controls given 0.5 ml isotonic saline and rats given 0.05 mg DFP twice weekly for 8 weeks, then 0.10 mg twice weekly for 2 weeks, then a final dose of 0.25 mg. The last injection was followed immediately by muscular tremors. This group of rats was injected starting the 5th day of diet and sacrificed on 76th day. No rat was sacrificed prior to 24 hours after an injection. Rats were weighed weekly. Red cell cholinesterase determinations were done by the method of Michel(12) on blood obtained from the tip of tail. Rats were killed by a blow on head, blood obtained by cardiac puncture, and samples of thigh muscle and liver were removed. The hematocrit; plasma water, sodium, potassium and chloride; and skeletal muscle and liver water, fat, potassium, sodium and chloride were determined according to previously described methods (13), except that sodium and potassium were determined by flame photometry.

Results. Body weight. After the first 10 days of potassium-deficient diet the rats had a decreased rate of gain in weight compared with those on normal potassium diet. Administration of DFP and Parathion did not alter rate of gain.

Cholinesterase activity. Fig. 1 shows red cell cholinesterase activity. Rats given Parathion had a moderate reduction in cholinesterase

terase activity and those given DFP had a marked reduction. There was no apparent difference between rats on a potassium deficient diet and those on normal potassium intake. Relative cholinesterase activity of intercostal muscle was estimated histochemically in Group A2(14). Cholinesterase activity at the motor endplate of potassium deficient rats showed no detectable difference from that of normals, but was definitely less in rats given DFP.

Hematocrits. There were no consistent differences among hematocrit values.

Plasma electrolytes. The potassium deficient diet produced marked hypokalemia and hypochloremia (Table I). There were no significant differences in the plasma electrolytes as a result of the administration of cholinesterase inhibitors.

Muscle water and electrolytes. Skeletal muscle of all groups showed essentially the same water content (Table I). Potassium deficient rats had a marked reduction of potassium in their muscles and a less marked increase in sodium concentration. No differences are found in muscle water or electrolytes between control rats and those given DFP or Parathion.

Liver water and electrolytes. Livers of potassium deficient rats tended to have a higher water content than did the normals (Table I). The reduction in liver potassium in potassium deficient rats was much less than the reduction in muscle potassium and the increase in sodium was slight. There were no apparent differences in liver water and electrolytes between control rats and those intoxicated with anticholinesterase compounds.

Derived data. Extracellular water phase, intracellular water phase, and intracellular sodium concentration are also shown in Table I. These have been calculated as presented by Cotlove *et al.* Changes in skeletal muscle due to potassium depletion, namely, increase in extracellular water, decrease in intracellular phase, and a marked gain in intracellular sodium concentration are similar to those reported by these authors(15). These changes do not occur in the liver. None of these derived values for muscle and liver discloses

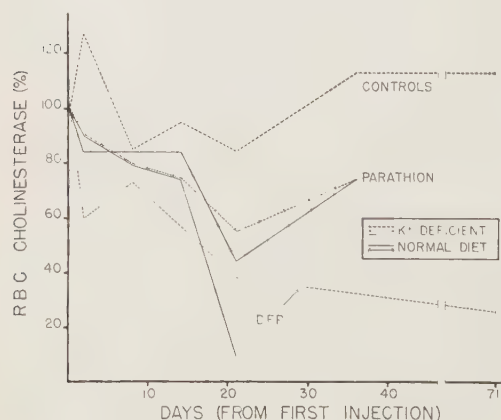


FIG. 1. Cholinesterase levels of red cells in rats given repeated injections of cholinesterase inhibitors (values expressed as % of the mean of 11 determinations on control potassium-deficient rats).

TABLE I. Water and Electrolyte Values in Plasma, Muscle and Liver of Control Rats and Rats Subjected to Cholinesterase Inhibitors.

Group	Plasma Determined				Muscle						
	(H ₂ O) g/l	(K ⁺)	(Na ⁺)	(Cl ⁻)	Determined				Derived		
		mEq/kg H ₂ O			(H ₂ O) g	(K ⁺)	(Na ⁺)	(Cl ⁻)	(H ₂ O) _E g	(H ₂ O) _C g	(Na ⁺) _C mEq
K deficient											
Saline control	923	2.34	161	104	764	57.0	52.7	13.3	112	652	36.5
S.E.	3	.09	2	4	1	1.7	3.2	.3	3	1	4.3
	(6)	(5)	(6)	(6)	(3)	(6)	(5)	(5)	(3)	(3)	(3)
DFP	925	3.09	156	101	768	63.7	48.1	13.1	118	646	29.4
S.E.	1	.49	2	3	2	2.6	3.9	.8	10	5	.8
	(6)	(6)	(6)	(6)	(3)	(6)	(6)	(6)	(3)	(3)	(3)
Normal K											
Saline control	919	6.51	158	111	763	103	17.6	11.9	102	671	.5
S.E.	3	.70	4	5	—	4	.9	.5	8	18	1.7
	(4)	(4)	(3)	(4)	(1)	(3)	(4)	(4)	(3)	(3)	(3)
DFP	919	6.45	154	117	764	98.3	18.7	10.0	92	672	4.8
S.E.	2	.31	2	2	3	3.8	.5	.9	7	7	2.9
	(4)	(4)	(3)	(4)	(3)	(4)	(4)	(4)	(3)	(3)	(3)
					Liver						
					Determined				Derived		
					(H ₂ O) g	(K ⁺)	(Na ⁺)	(Cl ⁻)	(H ₂ O) _E g	(H ₂ O) _C g	(Na ⁺) _C mEq
K deficient											
Saline control					734	72.9	24.5	20.0	177	557	-4.0
S.E.					4	4.8	1.4	1.4	11	9	1.5
					(4)	(5)	(6)	(6)	(4)	(4)	(4)
DFP					735	80.7	23.2	20.5	172	568	.5
S.E.					4	7.0	.7	.8	21	22	1.4
					(6)	(4)	(5)	(4)	(4)	(4)	(4)
Normal K											
Saline control					719	83.4	21.8	23.1	199	532	-.3
S.E.					3	8.8	1.9	1.7	10	21	8.2
					(3)	(3)	(4)	(4)	(4)	(4)	(4)
DFP					720	85.9	19.0	20.8	185	522	-6.8
S.E.					4	3.0	1.7	1.9	10	15	1.8
					(4)	(4)	(4)	(4)	(3)	(3)	(3)

Muscle and liver data/kg fat-free whole tissue.

S.E. = Stand. error of mean. Numbers in parentheses indicate No. of observations.

Data presented for Group A1 rats only.

any difference between animals receiving and those not receiving cholinesterase inhibitors.

Histologic study. Cardiac muscle revealed myodegeneration typical of potassium deficiency(16) and renal tubular changes of uncertain origin. No differences were noted between control animals and those given Parathion and DFP.

Discussion. Alterations reported here as the result of potassium depletion are similar to those found by Heppel(17) and Yannet and Darrow(18). Changes in water and electrolyte composition of plasma, skeletal muscle, and liver could not be related to repeated

administration of Parathion or DFP. Lack of evidence in these experiments that shifts of water and electrolytes occur as a specific result of cholinesterase inhibition suggests that hyperkalemia produced by acute lethal doses of anticholinesterases is unlikely to be related to changes in cellular permeability directly as a result of excessive concentrations of acetylcholine. The possibility remains that disturbance of cellular permeability may be too transient to have been encountered in these studies.

Summary. Normal rats and potassium deficient rats were injected with DFP and Para-

thion over many weeks. Electrolytes of plasma, skeletal muscle and liver of the potassium deficient rats showed the effects of potassium depletion. Studies of cholinesterase disclosed reduction in activity of this enzyme in rats receiving cholinesterase inhibitors. No effects due to cholinesterase inhibition were found upon either concentrations or distributions of water, potassium, sodium or chloride in plasma, skeletal muscle and liver.

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Effects of Adrenalectomy on Specific Activity of Deoxyribonuclease in Rat Spleen After Total Body X-Irradiation.* (22141)

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In view of the fact that specific activity of deoxyribonuclease II (DNase II)(1) in rat spleen is more than doubled after total body x-irradiation(2), it has been of interest to determine the role of adrenal cortex in this phenomenon. Leblond and Segal(3) recorded involution of the spleen as a result of localized x-irradiation after adrenalectomy. Prolonged administration of corticotropin to rats decreases cellularity of the spleen, and therefore the amount of deoxyribonucleic acid (DNA) present(4). Reduction in tissue

mass of the organ has been attributed to lymphocytolysis and the inhibition of mitosis resulting from adrenocortical steroids(5).

We have measured the DNase II activity in spleens of control, x-irradiated intact, adrenalectomized and x-irradiated-adrenalectomized rats.

Methods. Twenty-four young adult male rats of the Sprague-Dawley strain were used. They were divided into 4 groups of 6 animals each. The groups were treated as follows: Group I—control, Group II—x-irradiated-intact, Group III—adrenalectomized, Group IV—x-irradiated-adrenalectomized. All were housed in metal cages in an air conditioned room at 80°F, and fed a diet of Purina Lab-

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TABLE I. Effect of Adrenalectomy and/or 200 r of Total Body X-Irradiation on Average DNase II Activities of Rat Spleen.

Group No.	Treatment	γ DNA hydrolyzed/mg tissue/hr at 37°C	Spleen wt / Body wt ratio	γ DNA hydrolyzed/spleen/g body wt
I	Control (6)	14.09 $\pm$.98*	2.35 $\pm$.12 $\times 10^{-3}$	32.96 $\pm$ 2.64
II	X-irradiated intact (6)	26.71 $\pm$ 1.15	1.52 $\pm$.07 $\times 10^{-3}$	40.83 $\pm$ 3.54
III	Adrenalectomized (6)	13.76 $\pm$ 1.08	3.26 $\pm$.17 $\times 10^{-3}$	44.29 $\pm$ 1.56
IV	X-irradiated and adrenalectomized (6)	28.24 $\pm$.69	1.97 $\pm$.11 $\times 10^{-3}$	55.74 $\pm$ 3.76

* Stand. error.

Numbers in parentheses refer to No. of animals used.

oratory chow. They were given food and water *ad libitum*. Animals from Groups II and IV were adrenalectomized by sterile technique. Adrenalectomized animals were allowed 2 weeks for recovery, maintained on physiological saline solution rather than water for drinking. Animals from Groups II and IV were irradiated, using the following physical factors: 220 KV, 15 ma, inherent filtration only, HVL 0.25 Cu, 32 inches to center of animal, field size covering entire animal and at 35.5 r per minute in air for 5.6 minutes (200 r). Twenty-four hours later, animals from all 4 groups were weighed, stunned and killed by decapitation. Spleens were quickly dissected out, rinsed in distilled water, blotted dry on filter paper, and weighed. They were then homogenized in distilled water with an all-glass homogenizer, and homogenates diluted to give tissue concentrations of 20%. One-ml aliquots of homogenates were added to 1 ml portions of substrate solution, which contained 5 mg/ml of highly polymerized DNA[†] in a 0.1 M acetate buffer, pH 5, and these reaction mixtures were incubated in a water bath at 37°C for one hour. The reaction was stopped by addition of 2 ml of cold 10% trichloroacetic acid. After centrifugation and filtration, 2-ml aliquots of supernatant were reacted with Dische's diphenylamine color reagent(6). The violet color formed was measured on the Klett-Summerson photoelectric colorimeter.

Results. Results of the experiment indicate that adrenal cortex has no influence on the specific activity of DNase II/mg of rat spleen; that is, there is no significant dif-

ference between amounts of this enzyme activity per mg of tissue in control (I) and adrenalectomized (III) rats; nor is there any significant difference between x-irradiated intact (II) and x-irradiated adrenalectomized (IV) rats. However, there is a significant difference in total units of activity per spleen. Application of the "t" test(7) yields a P value for the change in total activity in groups I and III of $<.01$, in groups II and IV of $<.05$, in groups III and IV of $<.02$.

Results also confirm the fact that secretions of adrenal cortex have specific effects on involution of the spleen, as indicated by significant variations in spleen wt/body wt ratios in groups I and III, and groups II and IV in Table I.

Summary. Adrenalectomy has been found to have no effect on increased specific activity of rat spleen DNase II following total-body x-irradiation, but size of spleen and therefore total quantity of enzyme changed in both irradiated and unirradiated animals.

The authors wish to express their gratitude to Dr. I. Meschan, Professor of Radiology, for his kind assistance in irradiation of the animals.

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[†] Purchased from California Foundation for Biochemical Research, Los Angeles.

Qualitative Study of Aminoaciduria in Muscular Dystrophy and Myotonia Dystrophica.* (22142)

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Studies by Ames and Risley(1) indicated that increased amounts of amino acids were excreted in the urine of muscular dystrophy patients. However, no identifications of individual amino acids or excretion pattern studies were done. More recently Hurley and Williams(2), by means of quantitative paper chromatographic analyses, have reported higher leucine, taurine, threonine, valine and arginine levels in the urine of male patients. Glycine, alanine, serine and citrulline levels were not significantly higher than in normal urines. Urines of females were not analyzed.

In the present investigation an attempt has been made to discern the various amino acids excreted in the urine of male and female patients with muscular dystrophy and myotonia dystrophica by means of 2-dimensional filter paper chromatography. Siblings and parents of the muscular dystrophy patients were studied whenever possible.

Procedures and methods. The subjects were 43 muscular dystrophy patients ranging in age from 14 to 60 years, 9 male patients with myotonia dystrophica and 53 normal subjects. In addition, a group of 13 parents and 16 uninvolved siblings were investigated. Samples for analysis were taken from the first voided morning urine. All samples were analyzed the day of voiding or stored temporarily in the frozen state. The amino acids were determined by descending 2-dimensional filter paper chromatography, essentially by the methods of Levy and Chung(3) and Fink *et al.*(4). Aliquots of 70 microliters of urine applied to Whatman No. 1 paper in 3-micro-

liter portions were used for analysis. The developing solvents were (1) phenol-0.1 M borate buffer, pH 8.3 (85.6:20, w/v) and (2) butanol-glacial acetic acid-water (4:1:5) — organic phase. Development in phenol was carried out at $25 \pm 1^\circ$; and butanol at room temperature. Ninhydrin was used as the spray reagent(3). Because of similar R_F values, the following groups of amino acids could not be resolved: isoleucine and leucine; glutamine, citrulline and arginine; methionine and valine; and methionine sulfoxide and sarcosine. β -Aminoisobutyric and γ -amino-n-butyric acids if present would travel in the methionine-valine band. Taurine under these conditions is obscured by glycine in most instances. Further separations of these groups have not been attempted.

Results. There is a qualitative increase in the excretion of amino acids and certain related unidentified substances in muscular dystrophy patients, as shown by an increased number of spots on the urine chromatograms. The amino acids which appeared most frequently in the urine of dystrophy patients were methionine or valine, isoleucine or leu-

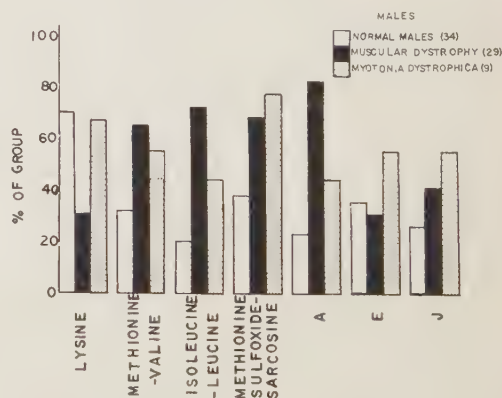


FIG. 1. Urinary amino acid excretion patterns in normal males and male patients with muscular dystrophy and myotonia dystrophica.

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† This work was done during the tenure of an Established Investigatorship of the American Heart Assn.

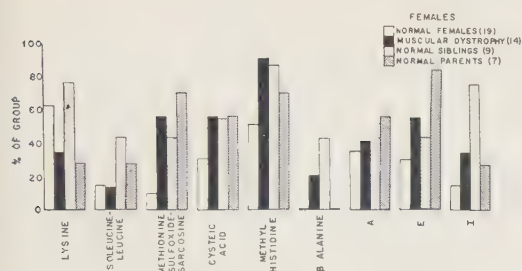


FIG. 2. Urinary amino acid excretion patterns in normal females, normal siblings, parents, and female muscular dystrophy patients.

cine, methionine sulfoxide or sarcosine, methyl histidine, cysteic acid and unidentified substances A and E (Fig. 1 and 2).[‡]

Nine subjects with myotonia dystrophica exhibited increased numbers of urinary amino acids. Lysine, methionine sulfoxide-sarcosine and unidentified substances E and J[‡] appeared more frequently in the urine of these patients than in the urine of muscular dystrophy patients (Fig. 1).

The amino acid excretion of a small group of clinically uninvolved male siblings and male parents was studied. There was a significant increase in the former group. Marked elevations of isoleucine or leucine, β -alanine, cysteic acid, and unidentified substances A and I[‡] were observed. In the male parent group, methionine-valine and cystine were increased (Fig. 3).

A group of uninvolved female siblings and female parents manifested marked increases in the number of amino acids excreted (Fig. 2). The aminoaciduria was as extensive in these latter groups as in the female muscular dystrophy patients.

A relatively large number of unidentified spots were observed in the chromatograms of muscular dystrophy patients and their relatives. Spots designated A and E were most consistently present.

Discussion. The data presented indicate that a qualitative increase in urinary amino acids occurs in patients with muscular dystrophy and myotonia dystrophica and certain

[‡] The R_F values of these unknown ninhydrin-reacting substances in solvents 1 and 2 respectively are— A: 0.75, 0.29; E: 0.50, 0.29; I: 0.85, 0.17; and J: 0.85, 0.11.

related normal individuals. Whether there is an associated quantitative increase in amino acid concentration in the urine of such individuals is currently under investigation.

The increased numbers of amino acids observed in the urine of normal siblings and parents of muscular dystrophy patients suggests the possibility of an inherited metabolic abnormality. The particularly large numbers of amino acids found in the urine of the normal female sibling and the maternal parent are striking evidence in favor of this thesis. Furthermore, genetic studies seem to indicate maternal transmission of the disease(6).

The presence of the relatively large group of unidentified ninhydrin staining substances, found in the urine of dystrophy patients and related individuals, is of interest. These components may be unidentified amino acids or peptides. It seems likely that these are normal constituents of urine, excreted at much lower levels in normal individuals since the urines of controls often contain these components. Boulanger, Biserte and Courtot(5) have described peptides in normal urines, some of which may be identical to those observed here. Two spots, A and E, are most consistently present in dystrophic individuals and their immediate relatives. The nature of these unknown substances is currently under study.

Summary. 1. An increase in the number of urinary amino acids and certain related unidentified substances was observed in muscular dystrophy and myotonia dystrophica patients. 2. Increased numbers of amino acids were found in the urine of normal sib-

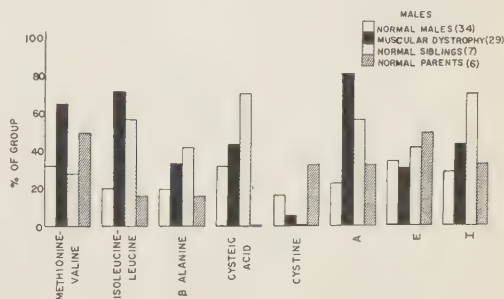


FIG. 3. Urinary amino acid excretion patterns in normal males, normal siblings, parents, and male muscular dystrophy patients.

lings and the maternal parents of muscular dystrophy patients.

The authors are indebted to Drs. Franz K. Bauer and Augustus S. Rose for their advice and encouragement throughout the course of this study.

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Effect of a High Fat Fructose and Casein Diet on Diabetic Cataracts.* (22143)

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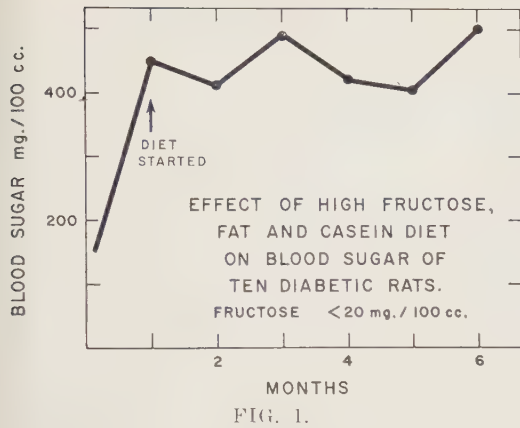
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Two or 3 months after the chemical induction of diabetes in rats, the lenses of the animals become opaque(1,2). The rate at which these cataracts develop is directly related to the height of the blood sugar(2). Decreasing the hyperglycemia by partial starvation(3) or the administration of phlorizin(4) delays or prevents cataracts to the extent that the blood sugar is lowered. These results suggest that hyperglycemia may play a direct role in the production of cataracts. If the high blood sugar level acts directly on the lens to produce cataracts, then the amount of sugar reaching the eye by way of the bloodstream should be of importance. Altering the blood supply by unilateral carotid ligation influences the development of galactose cataracts but does not affect the development of diabetic cataracts(5). This finding raises a question regarding the direct role of hyperglycemia and leads to the suggestion that the prevention of cataracts might be due to the provision of an alternate energy supply by the ketosis that accompanies phlorizin administration or starvation. Diets containing large

amounts of fat or protein provide non-carbohydrate substrates for energy and delay or prevent cataracts(1,6). However, these diets also lower the blood sugar and an interpretation of the results is difficult. Experiments in this laboratory indicate that a high-fat diet containing fructose rather than glucose, can be fed to diabetic rats without producing a depression in the blood sugar level. The use of this diet makes it possible to determine the effect of the dietary constituents in the prevention of cataracts. This paper reports the results obtained with a diet containing large amounts of fructose, fat and protein.

Methods. Male Sprague-Dawley rats weighing 76-173 g were made diabetic by the intravenous injection of 40 mg per kilo of alloxan monohydrate. The animals were maintained with unlimited amounts of water and ground dog chow. They were weighed and the blood sugars(7) determined once or twice each week. At the end of the first four weeks the blood sugar determinations for each rat were averaged. Those animals with blood sugar levels greater than 350 mg per 100 cc of blood were retained in the experiment and divided into two groups. The control group was maintained on the same diet as had been used in the first 4-week period. The experimental group was placed on an unlimited amount of the special diet. The latter diet consisted of 50% fructose, 25% corn oil and

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25% casein, fortified with an adequate amount of minerals and vitamins. Blood sugars were determined at least once each week and averaged for each 4 week period. During the next 4 months, 4 of the animals on the special diet had average blood sugars which fell below 350 mg per 100 cc of blood on one or more occasions. These animals were dropped from the experiment. Ten animals on the special diet and 10 receiving the chow diet maintained blood sugars consistently above 350 mg per 100 cc.

Results. The effectiveness of fructose in maintaining a high blood sugar level, in spite of a large amount of fat in the diet, is indicated by the blood sugar averages as depicted in Fig. 1. The blood sugar values represent the total amount of glucose and fructose in the blood. Separate determinations for fructose(8) on the same blood samples indicated that the fructose level was 10-20 mg per 100 cc.

The eyes of the rats were examined daily for cataracts as previously described(2). The control group developed cataracts between 59 and 120 days after the induction of diabetes with an average time of 73 days and a standard deviation of 18 days. The rats on the special diet were observed for a period equal to twice this average time or 150 days. During this period 2 of the 10 rats developed cataracts at 86 and 99 days. These cataracts occurred 2 and 3 weeks after a 3-day lapse in the special diet during which time the animals were fed a ground chow diet. The other

8 rats did not have cataracts at 150 days (Fig. 2).

Rats placed on the special diet showed a marked change in their growth rate. Before being placed on the special diet, the animals grew at the expected rate for diabetic rats having a blood sugar of 450-500 mg per 100 cc. After the initiation of the special diet, however, the growth rate was accelerated and comparable to that of diabetic rats with blood sugars between 250-300 mg per 100 cc. This was true in spite of the fact that the blood sugar of these animals was maintained at the higher level (Fig. 3).

Discussion. There are 3 effects of the high fructose, fat and casein diet which are worthy of further comment. First, it maintains a high blood sugar level. Second, it prevents the development of cataracts and third, it accelerates growth.

When a high fat diet containing sucrose is fed to diabetic rats, there is no glycosuria and the growth of the rats is stopped(1). In contrast, a high fat diet containing fructose results in a blood sugar comparable with that of a glucose low fat diet and the animals gain weight. It is possible that a high content of fat in the diet interferes with the intestinal absorption of glucose and not of fructose. Further studies are required to prove this point. The observation that there is very little fructose in the blood of a fructose fed animal is consistent with the fact that fructose is rapidly converted into glucose(9).

The fact that a high fructose, fat and casein diet prevents the development of cataracts definitely rules out hyperglycemia as a direct mediator in the production of cataracts and demonstrates that diabetic cataracts can

EFFECT OF HIGH FRUCTOSE, FAT AND CASEIN DIET ON DEVELOPMENT OF DIABETIC CATARACTS.

DIET	NO. RATS	AVE. WT. gm.	BLOOD SUGAR mg./100 cc.		PER CENT WITH CATARACTS AT 150 DAYS	
			CON-TROL	ON DIET		
					50	100
SPECIAL	10	125	450	448		P < 0.001
CHOW	10	129	490	475		

FIG. 2.

GROWTH ON FRUCTOSE, FAT AND CASEIN DIET.

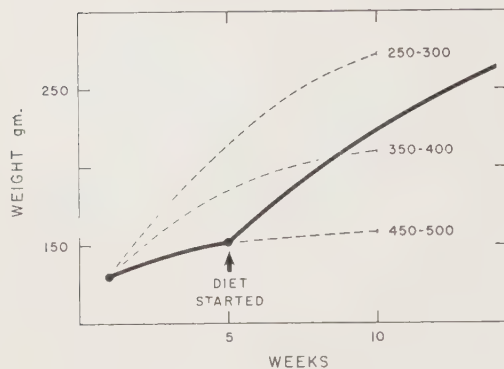


FIG. 3.

be prevented without insulin if energy sources other than glucose are made available. The results are consistent with the suggestion that diabetic cataracts are the result of an inability to obtain adequate energy by the utilization of glucose in the absence of insulin.

The growth rate of rats on a high fructose, fat and casein diet is accelerated. This indicates that tissues, other than lens, are benefited by a diet which provides substrates that do not require insulin for utilization. Rats on a high fat diet containing sucrose show no gain in weight over a period of three months (1). Thus, the presence of fructose provides an advantage. This may be due to the production of a high blood glucose level, and hence, a greater gradient for the penetration of glucose into the cells, or it may be due to the penetration of small amounts of fructose into the cell. It is doubtful whether this improvement in the distribution of carbohydrate is in itself responsible for the improved

growth rate. Diets containing fructose without fat (9) and high blood levels of glucose in the absence of fat do not accelerate the growth of diabetic rats. It is more likely that the improved distribution of carbohydrate increases the efficiency with which fat is utilized by the cell. This might be brought about by increasing the availability of oxalacetic acid which is essential for the complete metabolism of fat.

Summary. A diet consisting of 50% fructose, 25% corn oil and 25% casein and fortified with vitamins and minerals was fed to diabetic rats with blood sugar levels over 350 mg per 100 cc of blood. On this diet the blood sugar was maintained at a high level, cataracts were prevented and growth rate accelerated. The beneficial effects of this diet suggest that cataracts and the failure in growth of diabetic rats are the result of a deficit of energy resulting from inadequate glucose utilization in the absence of insulin.

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Growth of Spherules of *Coccidioides immitis* in a Chemically Defined Liquid Medium. (22144)

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The dimorphic fungus, *Coccidioides immitis*, undergoes a completely different type of growth *in vivo* from that *in vitro*. The parasitic form, or spherule, appears in animal tissue as a non-budding, spherical structure 20-80 μ in diameter with a thick, refractile, double wall, and is filled with numerous small endospores 2-5 μ in diameter. Reproduction takes place by rupture of the spherule wall, freeing the endospores, which increase in size and develop into typical large endospore-filled spherules. The saprophytic form appears in laboratory media as a mycelium with branching, septate hyphae which break up into numerous thick-walled, rectangular, ellipsoidal, or spherical arthrospores 2-4 μ in diameter. The arthrospores germinate and repeat the cycle. Observation of the parasitic phase in solid or semi-solid complex medium has been reported by several authors. MacNeal and Taylor(1) observed multiplication of spherules in infected pus when added to ascitic fluid or gelatinized horse serum containing sterile kidney slices. Lack(2) noted development of spherules from chlamydospores inoculated into Hall tubes containing glucose broth and partially coagulated egg albumin. Baker and Mrak(3) observed the development of spherules in a variety of solid media after 45 to 60 days, when active growth had ceased due to drying of the medium. Conant and Vogel(4) observed spherules on Sabouraud's dextrose slants following exposure of the cultures to Tween 80 (polyoxyethylene (20) sorbitanmonooleate). Burke (5) reported the appearance of spherules usually after 4 to 10 weeks, but occasionally after 11 days in a partially defined solid medium containing coconut milk.

Experiments in this laboratory resulted in the production of spherules in a liquid synthetic medium, following the observation of the parasitic phase in cultures grown in the presence of lithium hydroxide and tamol "N"

(an aryl sulfonic acid). The following presentation is devoted to a description of the medium, the development of the spherules, and the morphology of the cultures.

Methods and materials. 1. Inoculum used was a culture of *C. immitis*, strain M11, grown for 28 days at 34°C on a reciprocating shaking machine operating through a 4½ inch stroke at 96 strokes/minute. Growth medium for the inoculum was a glucose, acetate, inorganic salts medium, Basal #2, Roesler *et al.*(6) supplemented with 4 ppm Zn⁺⁺ as ZnSO₄. The inoculum (0.1 ml) was approximately 10 x 10⁶ viable arthrospores. 2. The experimental medium was a 5-fold dilution of the inoculum medium, containing .022 M glucose, .016 M ammonium acetate, .003 M each dipotassium and monopotassium phosphate, .0016 M magnesium sulfate, .0000124 M zinc sulfate and distilled water,

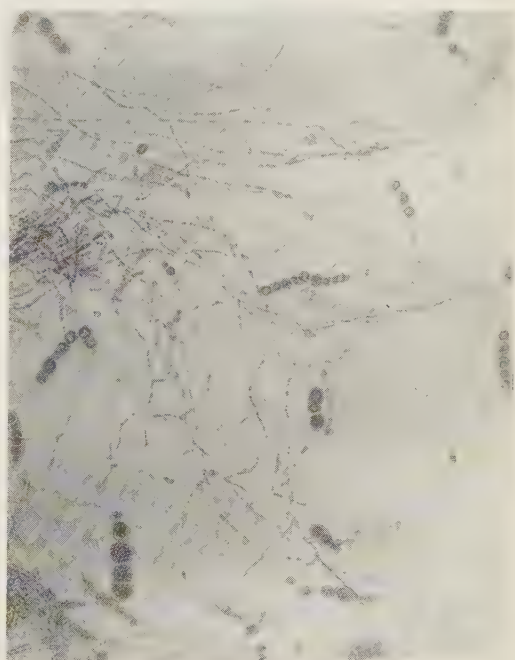


FIG. 1. Representative field of *C. immitis* culture showing growth of spherules, 200 $\times$.

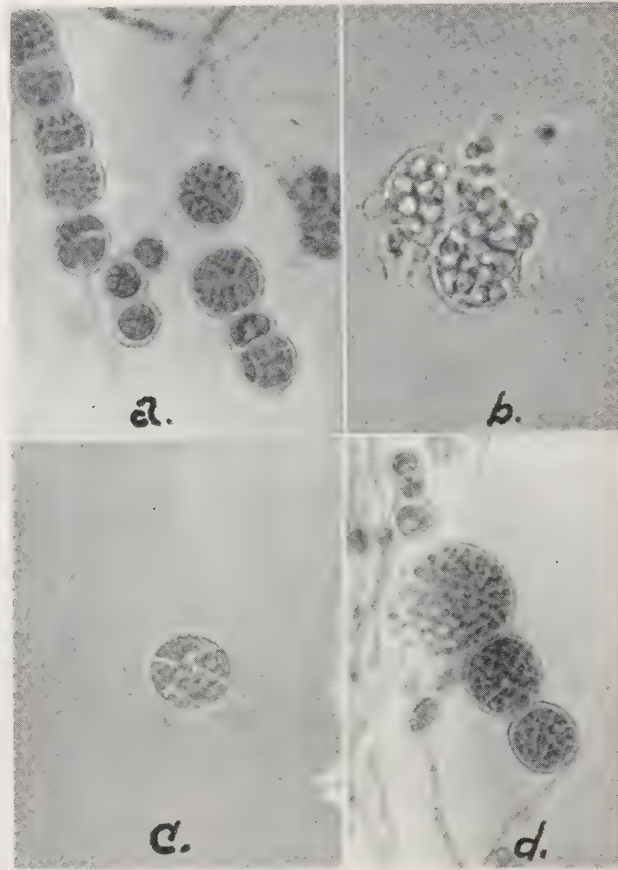


FIG. 2. (a) Spherules in various stages of development, 450 $\times$. (b) Two mature spherules with ruptured walls. Endospores are being extruded into the surrounding medium, 650 $\times$. (c) Young spherule showing developing cross-walls, 450 $\times$. (d) Chain of mature spherules. Note apparent rupture of wall of largest spherule and start of endospore release, 450 $\times$.

with final pH 6.6. This was dispensed into 250 ml Erlenmeyer flasks, 50 ml per flask, and autoclaved 15 minutes at 121.5°C. The flask closures were constructed from modified thistle tubes filled with gauze covered cotton and inserted through rubber stoppers. 3. Experimental cultures were incubated with shaking at 34°C and examined microscopically at 24-hour intervals. 4. *Evaluation.* Visual estimation of growth was made with unaided eye. Viable plate counts were made with a 1% peptone, 2% glucose, 0.1% autolysed yeast, 2% agar medium, with incubation at 34°C for 48 hours. Microscopic examinations were made on lactophenol-blue wet mounts. For photomicrographic purposes the edges of the cover slips on the wet mounts were sealed with Duco cement.

Results. (1) *Development of the parasitic form.* Spherules appeared after 72 hours incubation, following initial mycelial growth, with maximum production occurring between 96 and 120 hours. They occurred at terminal and intercalary locations of the hyphae similar to typical chlamydo-spore development on solid medium, and developed from chains of arthrospores, the typical spore-form of the *in vitro* type of growth in liquid medium. Upon continued incubation there appeared to be simultaneous germination of arthrospores into hyphae which produced both new arthrospores and spherules, and rupturing of spherule walls with the release of endospores which developed into new, mature, endospore-filled spherules. The parasitic form of growth appeared to comprise 50 per cent or more of the

total growth of the cultures.

(2) *Spherule morphology.* The spherules were morphologically similar to those occurring in animal tissue. They occurred as spherical or ovoid structures, 3 to 80 μ in diameter with double refractile walls, and contained varying numbers of endospores, depending on the size and stage of development of the spherules. The diameter of the endospores varied from 1 to 5 μ with the majority appearing to be one-tenth to one-fifth the volume of mature arthrospores which are normally 3 to 5 microns in diameter. The accompanying figures show representative fields of view and detailed morphology of a typical culture of *C. immitis* grown under the conditions described.

(3) *Other strains.* Although the majority of this work was accomplished with *C. immitis*, strain M11, a rodent isolate from Arizona contributed by Dr. C. E. Smith of Berkeley, Calif., 38 other strains isolated from humans and animals have been tested under identical conditions. Sixty per cent of these developed spherules in the experimental medium, but to a lesser extent than strain M11.

4. *Growth measurements.* The total growth of spherule cultures (15×10^6 per ml) in 5 fold diluted medium, as measured by viable plate counts, was approximately one-tenth that of arthrospore cultures (130×10^6 per ml) grown in the inoculum medium.

Discussion. Conditions for the growth and maintenance of the parasitic form of *C. immitis* in a chemically defined liquid medium are not yet fully understood although physical, chemical, and nutritional studies should throw further light on this subject. There seems to be a critical stage during the early development of the cultures at which time the

environment exerts its greatest effect in stimulating production of spherules. Harvesting the spherules must be done not later than the fourth or fifth day of incubation as a gradual decrease in numbers takes place thereafter, accompanied by a slow reversion of the cultures to the saprophytic phase. A possible explanation might be a selective change of environment involving the exhaustion of nutritional factors or the accumulation of metabolic products inhibiting growth of the parasitic form.

The use of a chemically defined liquid medium for production of spherules would materially benefit physical, chemical, and metabolic studies of the parasitic phase of the organism. It would also permit large scale production of antigenic material, free from foreign protein matter.

Summary. A method for growth of the parasitic phase (spherules) of *Coccidioides immitis* in a chemically defined liquid medium is described. A description of the development and morphology of the structures is included.

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Dissecting Aortic Aneurysm in Hamsters Treated with Cortisone Acetate. (22145)

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Dissecting aortic aneurysm has been produced in dogs by thermal coagulation of the aorta(1), and in rats by diets containing meal of the seeds of *Lathyrus odoratus*(2-4). No report has been found of the production of this aortic lesion in any other laboratory animals. During experiments in which hamsters were treated with cortisone to accomplish heterologous transplantation of human fetal lung tissue, many animals died with massive intrathoracic or intraperitoneal hemorrhage. Histologic examination of mediastinal and retroperitoneal tissues revealed that the hemorrhage was usually due to rupture of a dissecting aneurysm of the aorta.

Materials and methods. Seventy-seven golden hamsters 1 to 3 months of age, from the colony at the National Institutes of Health (NIH) were treated with cortisone acetate and inoculated with human fetal tissue. Fifteen additional animals were treated with cortisone but received no transplants. The fetal lung tissue was cut into small fragments and implanted with an 18 gauge trocar, or was minced with small quantity (usually less than 1 ml) of physiological saline and injected by using 1 ml tuberculin syringe and 20 gauge needle. Inoculations were made into submucosa of the cheek pouch of hamsters anesthetized with veterinary nembutal, 0.11 ml/100 g body weight. Cortisone acetate (Cortone,[®] Merck) was injected subcutaneously into the posterior cervical region in doses of 3 mg at the time of transplantation of human tissues, and 1 mg given twice a week thereafter for remainder of the life of the animal. Hamsters were kept in large plastic cages in air-conditioned rooms at approximately 78°F. The diet consisted of Purina Laboratory Chow and water *ad libitum*, supplemented with kale, carrots, and apples 3 times a week. Hamsters were examined daily, and dead animals necropsied im-

mediately. Six hamsters were killed when they appeared moribund. The other 71 were found dead, but had not appeared moribund on the preceding day. At first, only the cheek pouch area was examined histologically, since the state of heterologous embryonic tissue was our primary concern. When it was apparent, however, that many animals had died with massive thoracic or abdominal hemorrhage, an attempt was made to determine the origin of the hemorrhage. Entire thoracic contents were then removed at necropsy. Lungs were removed, and remainder of the specimen including thoracic aorta was fixed in Helly's formal-Zenker fluid. The abdominal aorta was also prepared for histological study. When hardened the tissues were cut transversely into 15 to 20 blocks. After tissues were imbedded in paraffin, sections were cut at 6 μ and stained with hematoxylin and eosin routinely. A variety of histochemical techniques has also been used, and these will form the basis for a later report.

Results. Massive hemorrhage was the cause of death of 51 of 77 NIH hamsters dying 26 to 162 days after initiation of cortisone therapy and transplantation of human fetal tissue. Of these 51 animals, 37 had hemothorax and 14 had hemoperitoneum. The aorta of 26 of these animals was examined microscopically, and dissecting aneurysm was demonstrated in 23. No inflammatory lesions were found. However, in sections from 3 other animals, necrotizing arteritis of the aorta was observed; this necrosis was so extensive that we were unable to determine whether dissection of the aorta had occurred. In addition, dissecting aneurysm was found histologically in 2 animals in which mediastinal hemorrhage had not taken place. Fourteen hamsters, 2 months old, obtained directly from Lakeview Hamster Colony in New Jersey were treated with

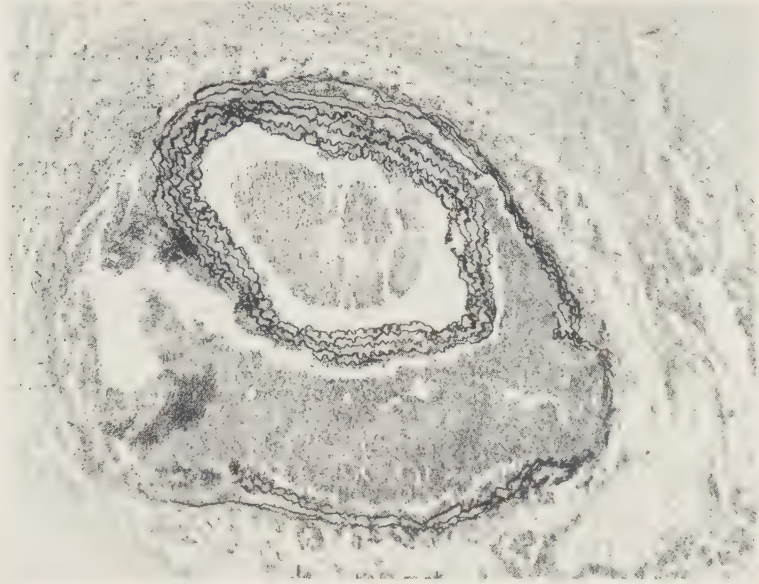


FIG. 1. Thoracic aorta of a cortisone treated hamster showing separation of elastic fibers by hemorrhage. Orcein. 60 $\times$.

cortisone acetate following implantation of pieces of human fetal lung tissue by the same technics as were applied to the NIH hamsters. Six of these animals died, all with hemothorax or hemoperitoneum. A control experiment to test the effect of cortisone alone is in progress. Five Lakeview and 10 NIH hamsters have been treated with cortisone acetate using the same dosage schedule, but without tissue transplantation. After 80 days 2 Lakeview and 4 NIH hamsters died with hemothorax or hemoperitoneum, and dissecting aneurysm has been demonstrated histologically in all.

Histologically, the aneurysmal dissection of aortic wall appeared to originate at a point lying just above aortic valve. The lesion consisted of separation of layers of the media of the aorta by extravasated blood (Fig. 1). In the sections of aorta from the 2 animals without massive hemorrhage, there was hyalinization of areas of the outer media adjacent to the aneurysmal vascular lumen. In animals with massive hemorrhage, medial necrosis was no longer evident histologically. The structural continuity of the blood-filled intramedial spaces with lumen of the vessel on the one hand or with perivascular tissue on the other, could be demonstrated in some

sections. Most of the aneurysms appeared to have developed acutely, since there was little evidence of inflammatory reaction in areas of dissection or hemorrhage.

Discussion. The factors involved in the degenerative lesion that eventuates in weakening of the tunica media and development of these dissecting aneurysms in hamsters, are not clear from these experiments. We have no evidence to suggest that this lesion is related to lathyrism. The diet contained no pea meal, although Purina Laboratory Chow does include legumes alfalfa and soybean. Gross examination has not shown striking abnormalities of bone which have been described in lathyrism(4). The diet has been fed to nearly a thousand other hamsters in our laboratory over long periods of time, and none have shown evidence of aneurysm. Water extraction of Purina Chow according to the method of Dupuy and Lee(5) has yielded an amorphous yellowish substance. The relationship, if any, between this material and the crystals isolated from *Lathyrus pusilis* awaits completion of biological tests now in progress. The possibility of a synergism between cortisone and some dietary factor is still to be considered.

No report of aneurysm in patients treated

with cortisone or corticotrophin has been found, although necrotizing arteritis was described recently following self-administered cortisone overdosage in an arthritic patient (6). Other investigators have found aortas of cortisone-treated children to have a high content of lipids (7). Degeneration of the tunica media of the ascending aorta without hemothorax has been observed in this laboratory in two mice bearing a subcutaneous transplant of a functional adrenal cortical carcinoma (8).

Summary. Massive thoracic or abdominal hemorrhage has been found in 51 of 77 hamsters treated for 26 to 162 days with cortisone acetate. All of these animals bore implants of human fetal tissue. Histological sections of 26 of these animals demonstrated dissecting aortic aneurysm in 23 and necrotizing ar-

teritis in 3 animals. The aorta of the other 25 animals with gross hemorrhage was not examined histologically. Six non-transplant-bearing hamsters treated with cortisone died with microscopically proved dissecting aortic aneurysm.

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Inhibition of Calcification *in vitro* by Luteocobalti Chloride.* (22146)

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There is evidence that chondroitin sulfate or its protein complex may play a role in the process of calcification. Rubin and Howard (1) found an increase in metachromatic staining in regions where active calcification is occurring, which they attribute to increase in concentration of chondroitin sulfate or a change in its degree of polymerization. Neuman (2) showed a correlation between cation binding capacity of cartilage and its sulfate content. Miller, Waldman and McLean (3) found that basic dyes inhibit calcification *in vitro* and that this effect can be reversed in the presence of calcium and phosphate. Finally, it has been demonstrated that protamine, which can precipitate chondroitin sulfate from solution, can cause inhibition of

calcification *in vitro* (4,5). Like protamine, the complex luteocobalti cation can precipitate chondroitin sulfate as shown by Matthews and Dorfman (6). It will also precipitate mucoprotein of cartilage (7), as well as ribonucleic acid, heparin and sodium polymetaphosphate, from solutions of low ionic strength and at a pH above 3.5 (6). Under similar conditions hyaluronate, neutral polysaccharides and human serum proteins fail to precipitate.

In view of this property of acting as a rather selective precipitant, it was decided to study the effect of luteocobalti chloride on calcification *in vitro*. It was also considered that the orange-yellow color of the cation might make it possible to detect its presence at calcification sites.

Methods. Calcification *in vitro* was studied by a simplification of the method described by Sobel, Nobel and Hanok (8). Twenty-one-day-old weanling rats were

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raised on a standard rachitogenic diet (U.S.P. Rachitogenic Diet No. 2). When they were 5 to 6 weeks old, they were sacrificed, the tibia was dissected out and sliced longitudinally. These tibial sections were placed in calcifying solution (described below) and incubated at 37°C for 16 to 20 hours. Sections were then washed with water, placed in 2% aqueous silver nitrate and exposed to the light of incandescent lamp for about 10 minutes. Calcification manifests itself as a dark deposit on the surface of the epiphyseal plate. It is measured by extent of this deposit, using numbers from 0 to 4, to designate width of surface of the plate covered and by zero or 1 to 4 plus signs, enclosed in parenthesis, to designate length of plate covered. Range of values extends from 0 (0) to 4 (++++). The action of luteocobalti chloride was studied in 3 ways; (1) incorporation of inhibitor into the calcifying solution itself, (2) prior exposure of sections to inhibitor in the basal salt solution, followed by washing with water and incubation in calcifying solution, and (3) prior exposure of sections to inhibitor in the presence of calcium chloride, followed by washing and incubation in the calcifying solution. The calcifying solution contained the following components: sodium chloride (0.07 M), potassium chloride (0.005 M), sodium bicarbonate (0.022 M), calcium chloride (0.0025 M; 10 mg% calcium), and a combination of NaH_2PO_4 plus Na_2HPO_4 at a molarity of 0.00161 corresponding to 5 mg% phosphorus. The pH of the solution was adjusted to 7.3 with carbon dioxide. The basal salt solution consisted of the following: sodium chloride (0.07 M), potassium chloride (0.005 M), and sodium bicarbonate (0.022

TABLE II. Inhibition of Calcification *In Vitro* by Prior Exposure to Luteocobalti Chloride.

Cone. of LCC (normality)	No. of exps.	Degree of calcification (avg)
.0	5	1(++++)
.0-(B.S.)	5	1 (+++)
.0011	2	$\frac{1}{2}$ (++++)
.0022	2	$\frac{1}{2}$ (+)
.003	2	0 (0)
.0056	4	0(0), 0(0), 0(0), 1(++++)
.011	4	0 (0)
.056	3	0 (0)
.112	3	0 (0)

B.S. = Basal salt solution.

M). The solution was adjusted to a pH of 7.3 with CO_2 .

Results. The results in Table I were obtained by addition of luteocobalti chloride to the calcifying solution in concentrations indicated. Inhibition began in the concentration range 8.96×10^{-3} to 1.9×10^{-2} N luteocobalti chloride. There was a deposition of cobalt ion on the surface of the epiphyseal plate increasing with concentration of luteocobalti chloride employed.

The results in Table II were obtained by prior exposure of tibial sections to luteocobalti chloride in the presence of basal salt solution. The system was shaken intermittently for $2\frac{1}{4}$ hours. Then the tibial sections were washed with water and placed in calcifying medium for 16 to 20 hours. The sample designated as zero normality of luteocobalti chloride is a control which received no prior treatment; B.S. indicates a control that received prior treatment with basal salt solution. Inhibition of calcification begins in the range 2.2×10^{-3} N to 3.4×10^{-3} N, if one ignores the single case of inhibition found at 5.6×10^{-3} N.

It has been demonstrated by Sobel, Nobel and Hanok(8) that inhibition of calcification *in vitro* by prior shaking with neutral salts may be reversed by subsequent shaking with calcium chloride. In view of this it was decided to determine the effect of calcium chloride on inhibitory action of luteocobalti chloride. Table III shows results of prior exposure of tibial sections to luteocobalti chloride in the presence of calcium chloride for $1\frac{1}{2}$ hours, followed by their exposure to calcifying solution.

TABLE I. Inhibition of Calcification *In Vitro* by Addition of Luteocobalti Chloride to Calcifying Solution.

Cone. of LCC (normality)	No. of experiments	Degree of calcification (avg)
.0	9	$1\frac{1}{2}$ (++++)
.0056	3	1 (+)
.009	2	1 (++)
.019	2	0 (0)
.028	4	0 (0)
.056	2	0 (0)
.112	7	0 (0)

LCC = Luteocobalti chloride.

TABLE III. Effect on Calcification of Prior Treatment with Both Luteocobalti Chloride and Calcium Chloride.

LCC (normality)	Cone. of CaCl ₂ (normality)	No. of samples	Degree of calci- fication (avg)
.00	.0	7	1½ (++++)
.00-(CaCl ₂)	.5	6	1½ (++++)
.0056	.5	4	2 (++++)
.011	.5	5	1½ (++++)
.0179	.5	4	1½ (++++)
.028	.5	5	1½ (++++)
.056	.5	4	½ (++++)
.112	.5	6	1 (++++)
.056	.001	6	1 (++++)
.056	.05	7	1 (++++)
.056	.10	5	½ (++++)

There is no complete inhibition of calcification. Calcium chloride appears to exert a protective effect on the calcifying system. The amount of cobalt deposited, indicated by depth of yellow color at the surface of epiphyseal plate, depended on the ratio of luteocobalti chloride to calcium chloride. The greater the concentration of the calcium chloride, the less the deposit of the cobalt salt. In this connection it was observed that precipitates, formed from luteocobalti chloride and chondroitin sulfate in aqueous solution, readily dissolved in the presence of calcium chloride.

The most probable binding site of luteocobalti chloride at cartilage surface is either the chondroitin sulfate or its mucoprotein. In view of the possibility of mucoprotein involvement, it was decided to determine the effect of trypsin on the binding of luteocobalti chloride by tibial sections. In these experiments tibial sections were exposed to a solution of Tryptar (lyophilized crystalline trypsin, Armour) at concentration of 10 mg/ml

in water for the times indicated in Table IV. The sections were then washed and exposed to luteocobalti chloride (.2%) for one hour. Evaluation of cobalt deposit was similar to that used in evaluation of calcification. However, due to the nature of the cobalt color, evaluation tends to be more subjective. The final value of the cobalt deposit was obtained by multiplying the number for length by the number for width of deposit. The results indicate a complete loss of capacity to bind luteocobalti chloride on exposure to trypsin for 75 minutes.

Discussion. The work reported here supports the view that chondroitin sulfate or a mucoprotein containing it may be one component of calcifying systems. The luteocobalti cation, to the extent that it is a relatively specific precipitant for chondroitin sulfate, may be more useful than other cations for studying reversible inhibition of calcification. The fact that nasal cartilage does not calcify *in vitro* under the test conditions, shows that a high content of chondroitin sulfate or its mucoprotein is not a sufficient condition to cause calcification.

Summary. 1. Luteocobalti chloride inhibits calcification *in vitro* when tibial sections are exposed to its action, either prior to their exposure to the calcifying solution, or concomitant therewith. The inhibition is antagonized by calcium ions. 2. That the luteocobalti cation deposits on the surface of the epiphyseal plate can be seen because of its color. This deposit is prevented by calcium ions. 3. The capacity of tibial sections to bind luteocobalti chloride is destroyed by their preliminary exposure to trypsin.

TABLE IV. Effect of Trypsin on Capacity of Epiphyseal Sections to Bind Luteocobalti Chloride.

Exposure time to trypsin (min.)	No. of sections	LCC deposit	Final value of LCC deposit
Control	1	3(++++)	12
15	1	2(++++)	8
37	1	1(++++)	4
45	2	0 (0)	0
50	1	½(++++)	2
60	1	½ (++)	1
75	2	0 (0)	0
100	2	0 (0)	0

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Absorption in Dogs Following Exclusion of Bile and Pancreatic Juice Using I^{131} Labeled Fat. (22147)

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For the past year this laboratory has utilized I^{131} labeled protein[†] (albumin) and fat[‡] (glycerol trioleate) in studying absorption and digestion in various disease states, *i.e.* pancreatic disorders, ulcerative colitis, small intestinal disorders such as sprue and Whipple's disease, and in post gastrectomy patients. In comparative studies using radioactive iodinated serum albumin and radioactive iodinated glycerol trioleate in dogs subjected to total pancreatectomy, there was a greater abnormality in digestion and absorption of the radioactive fat than in digestion and absorption of I^{131} labeled albumin as measured by percentage of radioactivity found in blood following the test meal containing these substances(1).

The present study was done to further evaluate the effect of exclusion of bile and pancreatic juice on the pattern of fat digestion and absorption by use of a fat test meal containing I^{131} labeled glycerol trioleate.

Materials and methods. Two types of fat meal were prepared, each identical except that one was emulsified in a Waring blender and contained 2-3 ml of polyoxyethylene sorbitan mono-oleate (Tween 80). The constituents of the meal were: $\frac{1}{2}$ ml of peanut oil[‡]/kg body weight of the animal, 10 μ c of

I^{131} labeled glycerol trioleate, the total mixed with equal volume of water. For 3 days prior to the test meal the animals were given .99 g tablet of potassium iodide daily to saturate the thyroid with iodine. All animals were fasted for 12 hours prior to the test. A rubber Levine tube was passed into the stomach of the unanesthetized animal and the test meal given via the stomach tube. 2 ml of venous blood was then drawn at 1, 2, 3, 4, 5, and 6 hours following the test meal into the stomach. Each sample of blood was then assayed for radioactivity in a calibrated sodium iodide thallium activated scintillation crystal well counter. Using measured radioactivity of the 2 ml sample and the calculated blood volume of animal (assumed to be 7.5% of body weight), the total radioactive content of blood was calculated and expressed as percentage of total ingested radio-iodine. From these data a curve was constructed showing the relationship of radioactivity in blood as a function of time. To establish a "normal" pattern of radioactivity of blood following a fat meal of peanut oil containing I^{131} labeled glycerol trioleate, 39 tests were done on 24 normal dogs. In 29 of these tests peanut oil and glycerol trioleate constituting the test meal were emulsified. Ten additional tests were done on 10 dogs in which the constituents were not emulsified. Ten dogs were then subjected to total pancreatectomy under sodium amytal anesthesia, following which they were given a diet as suggested by Markowitz (2) with daily injection of insulin. Two-three weeks following operation these animals

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† Obtained from Abbott Laboratories, Chicago, under A.E.C. authorization.

‡ Obtained from Planters Nut and Chocolate Co., Wilkes-Barre, Pa.

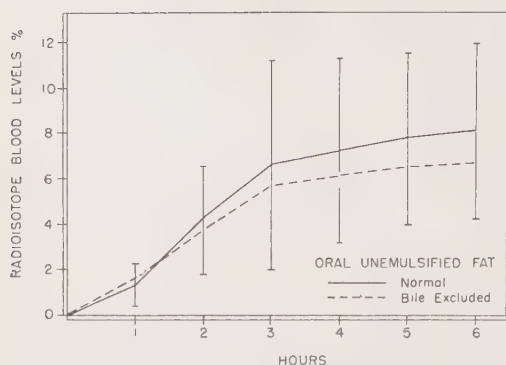


FIG. 1. Graph showing comparison in mean curve of blood isotope percent in 10 normal and 10 bile duct occluded dogs using an I^{131} labeled unemulsified fat test meal.

were tested with the peanut oil and I^{131} labeled trioleate emulsified meal. Fourteen additional dogs had ligation of their common bile duct and two weeks later were tested with the emulsified fat meal. Ten of these duct ligated animals were tested a week later with the unemulsified fat preparation.

Results. The results revealed no statistical difference between normal dogs and dogs with bile exclusion tested with I^{131} labeled unemulsified fat (Fig. 1). Likewise, there was no significant difference between normal and bile duct occluded dogs tested with I^{131} labeled emulsified fat (Fig. 2). There was, however, a striking difference in radioisotope blood level in normal and in pancreatectomized dogs tested with I^{131} labeled emulsified fat (Fig. 2).

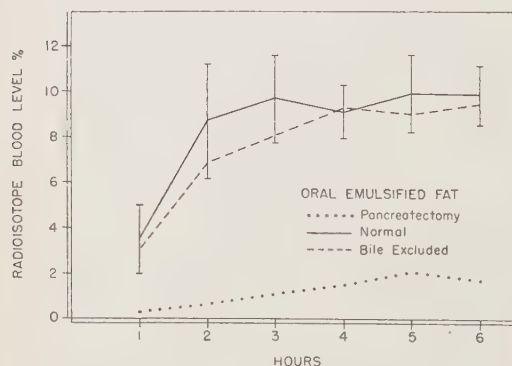


FIG. 2. Graph showing comparison in mean curve of blood isotope percent in 14 normal, 14 bile duct occluded, and 10 pancreatectomized dogs, all tested with an I^{131} labeled emulsified fat test meal.

Previous studies, using different technics have indicated that bile is quantitatively concerned in the digestion and absorption of fat (3-5) and that exclusion of bile reportedly results in decreased fat absorption (6). Further, previous studies have indicated that exclusion of pancreatic secretion produces a more marked alteration of fat digestion and absorption. Several investigators have used an iodized fat in studying fat digestion (7-10). Earlier studies (11-13) showed that although an iodized fat is unnatural, its digestion, absorption and metabolic fate closely followed that of neutral fat after oral administration. The glycerol trioleate used in the present study is a long chain, predominantly saturated fat. The peanut oil is a mixture consisting of mainly unsaturated fatty acids of high iodine index. From a chemical standpoint the bond between the I^{131} and the fat molecule theoretically is a firm one. Although the exact nature of fat digestion and absorption is not known, one theory holds that when a fat, as used in this study, enters the normal gut, hydrolysis of the fat molecule occurs, yielding glycerol and fatty acids, to which the I^{131} remains bound. Glycerol and fatty acids supposedly then re-unite in the wall of the gut to form again a fat molecule before entrance into the lacteals. Studies in this laboratory, to be reported elsewhere, show that undigested portion of administered fat passes in the stool, carrying with it the equivalent proportion of bound I^{131} . Furthermore, pancreatectomy had no influence on rate of absorption of ionic I^{131} , but has a profound influence on the rate of entrance of I^{131} into blood when it is chemically bound to the fat molecule.

The present study indicates that addition of an emulsifier produced little or no effect on fat digestion and absorption in the normal animal. Exclusion of bile made little difference in rate and in total amount of radioactive labeled fat entering the blood during the 6-hour period of the test, whether the fat was emulsified or unemulsified. On the other hand, exclusion of pancreatic juice caused a marked depression in the amount of radioactive labeled emulsified fat which entered the blood during the test period.

Summary. Using a fat test meal containing a radioactive labeled fat, a study has been carried out in dogs to assess the rate and total amount of radioactive labeled fat entering the blood in normal dogs and in dogs with exclusion of bile and pancreatic juice during a 6-hour period after administration of the meal. These studies indicate that bile plays a minor role and pancreatic juice a major role in fat digestion and absorption. This study suggests the use of a similar test for clinical use in evaluating pancreatic function in suspected pancreatic disease.

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Experimental Hydro-uteri (Hydrometra) in Rodents and Some Factors Determining Their Formation.* (22148)

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In mice of the English Silver Strain, and occasionally in other strains there occurs a condition of imperforate vagina which leads to a marked distention of uteri with accumulation of fluid up to 15 ml or more per mouse. Yet normal function of the uterus is preserved, since such animals become pregnant if the membrane occluding the vagina is removed(1). Existence of huge hydro-uteri in the presence of imperforate vaginas suggested that simple ligation of the cervix in rodents might likewise induce formation of hydro-uteri. This new technic not only provides a large uterine receptacle for tumor transplants but also permits the collection of large

amounts of uterine secretions in small animals.

This paper reports studies on production of hydro-uteri in mice, rats and hamsters and on hormonal and other factors which play a role in development of experimental hydro-uterus.

Methods. The cervix of the uterus was ligated under nembutal anesthesia through a supra-pubic incision in 456 mice, in a smaller number of rats (Wistar) and Syrian hamsters. The variables for this study of effects on development of hydro-uteri were strain of mouse, endocrine status and time elapsing between uterine cervix ligation and autopsy. The strains of mice and types of animals used are shown in Tables I and II. Eighty-four mature white Swiss mice, 40 DBA/1 and 40 C57BL/10J mice were castrated and their cervixes ligated (Table I). Forty-seven immature intact white Swiss mice underwent

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TABLE I. Hydro-uterus Formation in Immature and in Castrated Animals.

	No. of animals	Strain	Age, mo	Duration of ligation (D)	% hydro-uteri		
Intact immature animals	47	Wh. Sw. Tufts	1½-1	13-28	21		
Castrated mature animals	44	Wh. Sw. Tufts	3-8	15-60	40		
					Castrate control	Testosterone (.6 mg)	Estradiol (12 γ)
Castrated mature animals	40	Wh. Sw.	2½	21	0	0	30
	10*	RJB stock					
	40	DBA/1	2½	21	0	0	20
	10*	RJB stock					
	40	C57BL/10J	2½	21	0	0	20
	10*						

* In each category.

TABLE II. Hydro-uterus Formation in Intact Mature Animals.

	No. of animals	Strain	Age (mo)	Duration of ligation (D)	% hydro-uteri
Mice	163	Wh. Sw. Tufts	3-12	21-180	33-(66)
	51	BBFl	3-7	21-190	59-(90)
	12	C57BR/a	6	24	33
	35	Wh. Sw. RBJ stock	2½	22	20
	42	C57BL/H-2d	2½	42	29
	26	DBA/1 RBJ stock	3	30	35
Rats	10	Wistar rats	4	22	30
Hamsters	1	Golden Nugget	2	35	Positive

cervical ligation. One to 2 weeks later, castrates received injections subcutaneously as follows: *controls*, 0.5 ml sesame oil; *estrogen treated animals*, 1 γ estradiol daily; *testosterone treated animals*, 0.1 mg testosterone propionate every other day for 12 days. Twelve days later these animals were sacrificed. The effect of length of time after ligation of the cervix was studied in mature intact animals by performing exploratory laparotomies at varying intervals (up to 12 months) after cervical ligation. At the end of the experiments the animals were sacrificed and histological studies made of the uteri. The fluid which accumulated in the uteri was harvested and analyzed[†] for the following constituents by the indicated methods: for proteins(2),

carbohydrates(3,4), hexosamine(5) and hexuronic acid content(6). Bacteriological and cytological studies were also carried out. For purposes of comparison fluid from the hydro-uterus of a white Swiss mouse with imperforate vagina, ascitic fluid from Ehrlich's ascites tumor growing in white Swiss mice and from a foreign body ascites provoked by intraperitoneal injection of Walker (rat) Sarcoma cells into the abdomen of mice was similarly analyzed.

Results. The data obtained are summarized in Tables I, II and III.

Hydro-uterus formation in intact mature animals. In Table II approximately one-third of all animals which had undergone ligation of their uterine cervix developed distended uterine horns containing at least 0.5 ml of fluid 21 days after ligation. It is not clear why the remaining two-thirds of these

[†] We are indebted to Drs. W. H. Fishman and J. Nisselbaum for these analyses.

TABLE III. Chemical Composition of Various Hydro-Uterine and Ascitic Fluids.

Fluid pools studied	Protein content(2) (mg/ml)		Carbohydrates in the phosphotungstate precipitate		
	Ppt'd by HClO ₄	Ppt'd by phosphotungstate	Carbohydrate content (3,4) (mg/ml) expressed as half mannose, half galactose	Hexosamine(5) content (mg/ml)	Hexuronic acid(6) content (mg/ml)
HF* from WSM No. 4 7 mo after uterine ligation	trace	12.2	3.8	2.5	.15-.25
HF from WSM No. 4 11 mo after uterine ligation	trace	5.2	1.6	.77	.4 -.5
HF from mouse with imperforate vagina	30.0 (approx. values; total ls 63.5 mg/ml)	33.5	2.8	3.6	
AF from Ehrlich Ascites tumor	52.2	2.9	.24	1.2	0
AF from ♂ WSM with Walker tumor abdom.	34.2	1.5	.13	.6	.17

* HF = Hydrouterine fluid; WSM = White Swiss mouse; AF = Ascitic fluid.

animals fail to accumulate fluid in distended horns and show reasonably normal uterine horns in spite of cervical ligation. The possibility that this is merely a result of chance variations in the surgical technic can not be ruled out. Simultaneous ligation of the 2 oviducts in 136 mice and 7 rats did not increase significantly the frequency of hydro-uteri. The most important single factor favoring development of hydro-uteri is time, since in 2 groups of 12 animals, studied for 6 months, from 66 to 90% showed large hydro-uteri (Fig. 1). Fluid continued to accumulate rapidly in spite of periodic emptying of the hydro-uterus by aspiration of the fluid by needle puncture.

The tendency to formation of hydro-uterus following cervical ligation is not clearly determined by genetic factors since non-inbred white Swiss mice (Tufts), inbred white Swiss mice (Jackson, C57Br/a, C57BL/H-2d and DBA/1 mice all behaved essentially in the same way. It appears, however, that the BBFl mice tend to have a greater incidence of hydro-uteri under otherwise similar experimental conditions. Ligation of the cervixes of rats produced the same results as in mice. A hydro-uterus was also obtained in a hamster.

Chemical analysis of various hydro-uterine fluids yielded results shown in Table III. It appears that the protein content (precipitated

by HClO₄) of hydrouterine fluid in experimental hydro-uteri is considerably lower than that in ascites fluids and that the mucopolysaccharide content as reflected by the phosphotungstate precipitable proteins and carbohydrates of the phosphotungstate precipitate is higher. In fluid collecting in the uterus with imperforate vagina the protein content is higher than in ascites fluid. Bacteriological studies showed that all fluids studied were sterile.

Studies on the effects of hormonal factors are shown in Table II. Within the same period of time immature white Swiss mice showed the same incidence of hydro-uteri as mature intact animals. Castrated white Swiss mice observed for the same length of time (21 days) had no hydro-uteri, but after 60 days formed as many or more hydro-uteri as did intact controls (Tables I and II).

In other strains of mice, namely in inbred white Swiss mice, DBA/1 and C57BL/10J mice, castration prevented formation of hydro-uteri but they formed again when estradiol was given. However, it was found that if the castrated, untreated animals were again examined 2 or 3 months after ligation, many had formed hydro-uteri. Castration merely slows development of hydro-uteri but does not prevent it. Testosterone did not prevent or alter the retarding effect of castration, but

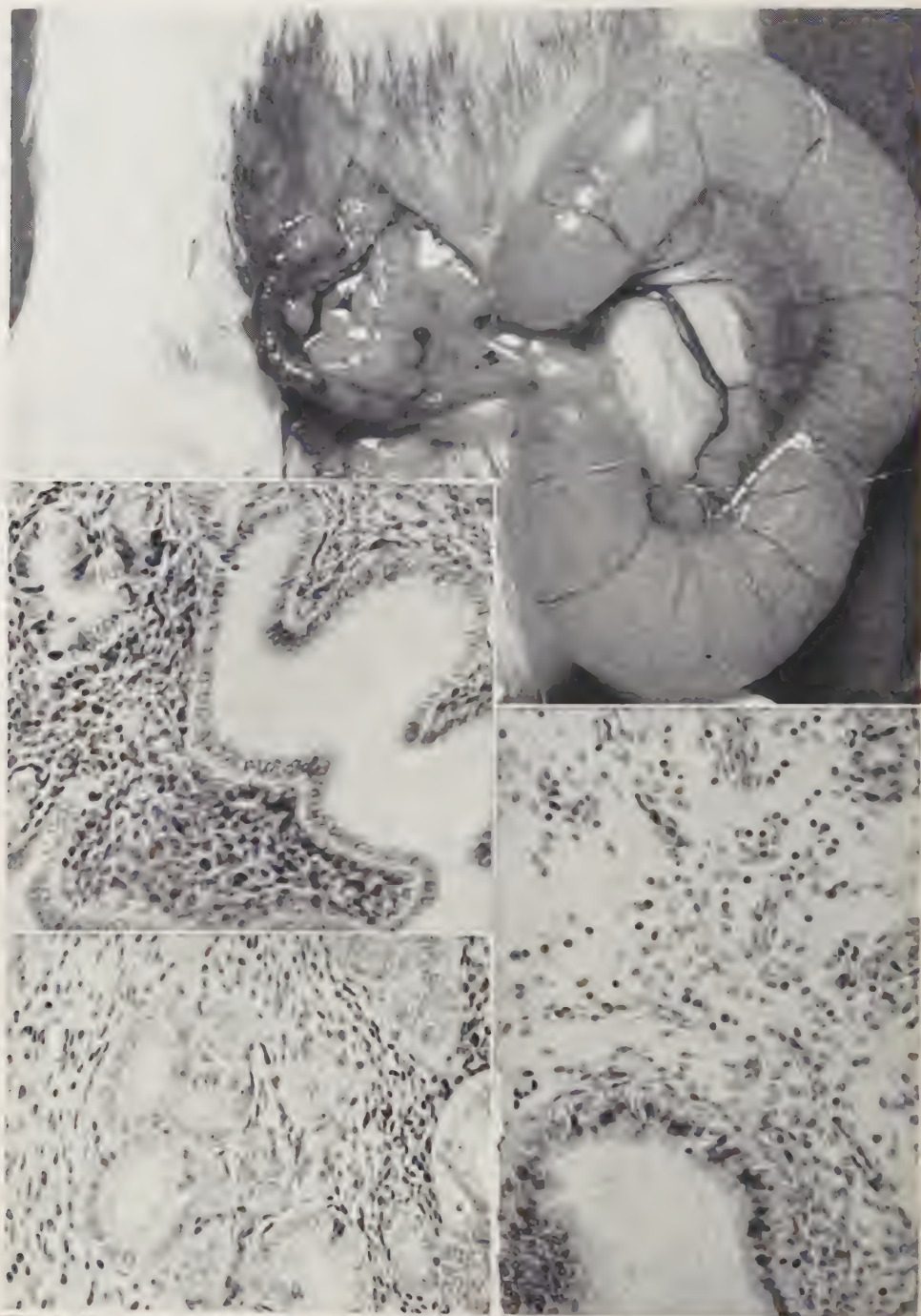


FIG. 1. Top: Appearance of unilateral hydrometers in a white Swiss mouse, 9 mo of age, 8 mo after ligation of left uterine horn, near cervix. Compare size of hydrouterus with that of non-ligated horn. Left center: Microphotograph of hydrouterus in normal control animal, 21 days after ligation. Left bottom: Microphotograph of hydrouterus in estrogen treated castrate, 21 days after ligation (same magnification). Right bottom: Microphotograph of hydrouterus in testosterone treated animal, 21 days after ligation (same magnification).

estradiol given to castrates restored the rate of formation of the hydro-uterus.

Histological examination of uteri after cervical ligation showed that the wall of the uterus, the endometrium and the endometrial glands react normally to stimulation by estrogens (Fig. 1). Testosterone produced atrophy of the glandular elements of the uterus and edema of the uterine wall with rarification of its cellular elements (Fig. 1). In all instances studied histologically there was complete absence of any inflammatory reaction in the uterine wall.

Discussion. Ligation of the cervix of mice, rats and hamsters leads to accumulation of a fluid in the uterus which can be harvested by uterine puncture and which keeps accumulating after each consecutive tap. As much as 100 ml of hydro-uterine fluid has been obtained from a single mouse during a year's time. The hydro-uteri provide an ideal site for tumor growth(7).

Endocrine imbalance produced by castration retards formation of hydro-uteri but does not prevent it, and administration of estrogen restores the rate of accumulation of intra-uterine fluid to normal. In immature mice the incidence of formation of hydro-uteri is reduced to about one-half of that seen in nor-

mal mature animals of the same strain.

Characteristics of hydrouterine fluid are different from those found in ascitic fluid, since the protein content is low and carbohydrate is high. This feature of hydrouterine fluid is compatible with the characteristics one would expect from a collection of endometrial secretions. If the collecting fluid is allowed to accumulate for several months, the relative proportions of its main components do not change but their concentrations are lowered, indicating some dilution taking place with time. It is believed that the experimental production of hydro-uteri in rodents constitutes a new method for the collection of endometrial secretions.

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Estimation of Bromide on Fingertip Blood in Bromide Intoxication. (22149)

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On several occasions it has been necessary to follow bromide levels of serum of small infants after bromide administration either therapeutically or accidentally. The method usually employed (bromide as gold bromide) requires amounts of serum greater than is conveniently obtainable from capillary blood. This paper describes a procedure practical for fingertip blood. The gold bromide procedure is used except that readings are taken in ultraviolet where a higher absorption peak exists for gold bromide.

Method: *Reagents and materials.* The

contents of a one g vial of gold chloride ($\text{AuCl}_3 \cdot \text{HCl} \cdot 3\text{H}_2\text{O}$) 0.25%, is washed into distilled water and made up to 400 ml. Trichloroacetic Acid: 20 g is made up to 100 ml with distilled water. Carbon Tetrachloride: Analytical reagent grade. Bromide Standard (100 mg/100 ml): 641 mg of sodium bromide (dried overnight in vacuum desiccator) is made up to 500 ml with distilled water. Screw Cap Tubes: Kimble #45066, 16 x 125 mm, fitted with screw caps, teflon lined, are rinsed with dilute nitric acid, distilled water, and dried.

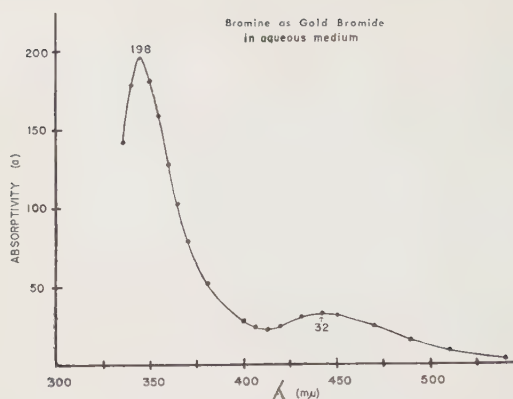


FIG. 1. Absorption spectrum of gold bromide in trichloroacetic acid, under conditions of the procedure described. Beckman spectrophotometer.

Procedure: Add 0.2 ml distilled water to screw cap tube. Wash in 0.05 ml of unknown serum. Add 1.3 ml of 20% trichloroacetic acid, mix. Follow with 1 ml carbon tetrachloride. Cap the tube and shake vigorously by hand or shaking machine for a few minutes and centrifuge 5 minutes at 2000 r.p.m. in a #1 International centrifuge or its equivalent. Transfer 1 ml aliquot from top layer to the quartz cuvet (1.5 ml capacity, 1 cm light path)*. Add 0.2 ml of 0.25% gold chloride, mix and read on Beckman Spectrophotometer at 350, or 440 $m\mu$ if optical density is greater than 0.8. For the standard, use 0.05 ml of standard instead of serum. For the blank, use 0.05 ml distilled water instead of serum. Calculations: Read bromide concentration off standard curve. For clinical purposes the formula below will yield sufficiently accurate results:

$$\frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times 100 = \text{mg/100 ml bromide in unknown serum}$$

Results. Fig. 1 illustrates the absorption curve of gold bromide which exhibits a peak at 350 $m\mu$ and at 440 $m\mu$. The latter is the peak customarily employed(1,2). By reading in the ultraviolet an increase in sensitivity by a factor of approximately 6 is obtained.

The concentration of gold affects the shape of the absorption spectrum. At higher gold concentrations the peak shifts toward the higher wave lengths. If the gold concentra-

tion is doubled the ultraviolet peak moves to 355 $m\mu$. The peak at 440 $m\mu$ is not affected by changes in gold concentration.

Location of the lines in Fig. 2 were obtained by applying the method of least squares(3) to 6 determinations, at each level shown, to find the line of best fit. It is to be noted that both at 350 $m\mu$ and at 440 $m\mu$ the lines do not go through the origin. Thus the method may not be used for normal serum where concentrations are of the order of 0.1 mg bromide per 100 ml(4).

A line may be drawn through the origin passing near the points. Thus the Beer-Lambert law may be applied, approximately, when dealing with bromide intoxication, since the toxic level is above 80 mg bromide/100 ml serum(5).

Gold chloride and bromide complex is extractable into higher alcohols and ketones at strongly acid pH but not into other solvents such as chloroform, benzene or heptane. Fig. 3 shows absorption spectrum of gold bromide

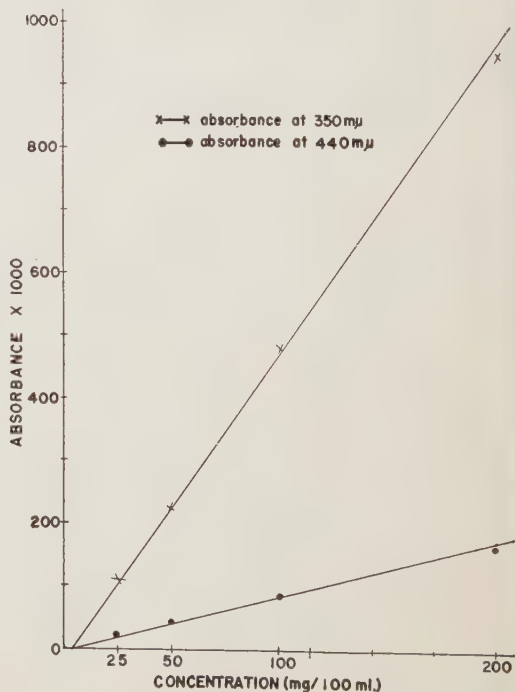


FIG. 2. Calibration curve plotting concentration vs. absorbance. Each point is the mean of 6 determinations. Position of line calculated by method of least squares. Concentration refers to concentration of the serum.

* Pyrocell Mfg. Co., N. Y. City.

extracted into octyl alcohol from a 10% sulfuric acid solution. Attempts to extract the bromide quantitatively, without first precipitating proteins, were not successful, and this method, then, had no advantage over the aqueous method for serum or plasma analysis. This procedure would find application when small amounts of bromide are present in large aqueous volumes, the volume of octyl alcohol layer is then limited to a small volume.

Filtrates with trichloroacetic acid tend to be cloudy, occasionally. This is true especially when serum is first diluted with water before adding trichloroacetic acid. This is avoided by adding carbon tetrachloride when precipitating the proteins as described in the procedure.

Table I shows recoveries of added bromide to a pooled sample of serum at different levels. High recoveries for the lower quantities are probably due to substances in the centrifugate other than bromine reacting with the gold chloride. In the high range, disappearance of appreciable amounts of gold lowers recovery values. In these high ranges it is

TABLE I. Recovery of Bromide Added to Serum.

Wave length (m μ)	μ g added	μ g found*	% recovery
350	12.5	13.1 $\pm$ 1.8†	104
"	25	26.6 $\pm$ 2.3	104
"	50	51.2 $\pm$ 4.4	102
"	100	98.5 $\pm$ 6.5	98.5
440	25	26.1 $\pm$ 3.4	104
"	50	51.1 $\pm$ 4.9	102
"	100	97.0 $\pm$ 5.8	97

* Six determinations at each level.

† Mean $\pm$ stand. dev.

suggested that smaller amounts of serum (0.02 ml) be used. The results at 350 m μ compare with those at 440 m μ , indicating that readings at 350 m μ permit estimation of smaller quantities of bromide without loss in precision. When absorbance at 350 m μ is higher than 0.800, then one may read at the less sensitive peak, 440 m μ . This permits a wide range of concentrations to be read without the need of repeating the analysis with smaller quantities.

Summary. A procedure is described for bromide analysis in serum of patients with bromide intoxication which uses the ultraviolet absorption peak for gold bromide to increase sensitivity. This permits the analysis of serum or plasma, from fingertip blood, for bromide analysis.

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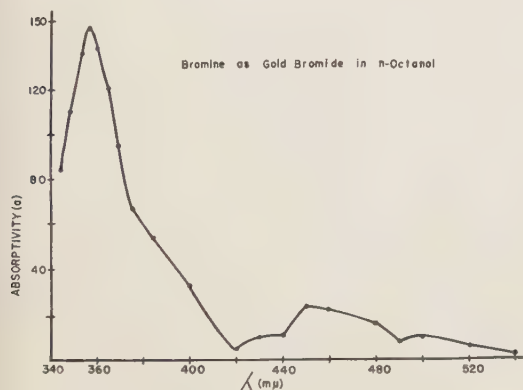


FIG. 3. Absorption spectrum of gold bromide extracted from 10% sulfuric acid into n-octanol. Beckman spectrophotometer.

Histamine Sensitivity of Adrenalectomized and Pertussis-Treated Mice. (22150)

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It has been established that mice, which are normally resistant to histamine, become highly sensitive after being injected with *Hemophilus pertussis* vaccine (see refs. in 1-10). The mechanism by which this vaccine increases the sensitivity to histamine by 25 or 100 times remains unknown, although several authors have suggested that diminished function of adrenal cortex and medulla may be involved since adrenalectomized mice are known to be sensitive to histamine (usually injected intraperitoneally) (10-14), and sensitivity is partially or completely lost after treatment with epinephrine and cortisone (5,14). It seemed pertinent to make a quantitative study to determine the degree of histamine sensitivity which develops in mice following treatment with pertussis-vaccine as compared directly with the sensitivity following adrenalectomy. The results of this study revealed that the sensitivity to histamine induced with pertussis vaccine could scarcely be related to altered function of the adrenal gland since adrenalectomized mice of the strain used were no more sensitive to intravenous injections of histamine than normal mice, whereas pertussis treatment of intact mice enhanced sensitivity to histamine 33 times.

Methods. The general plan of the experiment was to determine concurrently the LD₅₀ of histamine diphosphate in normal, adrenalectomized and pertussis-treated mice in order to make a direct comparison of histamine sensitivity in the 3 types of animals. Female albino mice of the Swiss-Webster strain (Taconic) weighing between 15 and 27 g were obtained from a local source, and maintained before and after operation or vaccine injection on a diet of Purina Laboratory Chow. Bilateral adrenalectomies were conducted under light ether anesthesia and operated mice were provided

with 0.9% saline until injected with histamine the following day. All other mice were supplied with tap water *ad lib*. *Hemophilus pertussis* vaccine supplied by the Massachusetts State Health Department contained 20 billion Phase I organisms per ml and Merthiolate in concentration of 1:10,000. This was diluted with saline so that each 0.5 ml contained 8.75 billion organisms, this volume being injected intraperitoneally in mice 4 days prior to histamine injection. Mice in control groups were intact and untreated at the time of histamine injection. Histamine diphosphate solutions were prepared in normal saline, then several dilutions in saline were made to obtain concentrations which permitted injection of various doses (Table I) in constant volume of 0.01 ml per gram of mouse. Histamine solutions were refrigerated and used within 48 hours of preparation. Injections of histamine were made into the tail vein during a period of 10 seconds, 4 graded doses being injected into normal, adrenalectomized and pertussis-treated mice on each day of experimentation. Each dose of histamine was injected into 20 mice, with exceptions as indicated in Table I.

Completeness of adrenalectomy was checked by providing a few mice from each operated group with 0.9% saline for 24 hr and tap water thereafter, incidence of mortality being recorded during 14 days. Some of these mice were fed the Purina Chow, others were fed oatmeal which has a lower sodium content. Histamine injections were made in adrenalectomized mice 24 hr after operation in order to minimize the possibility of growth of adrenal rests. Incidence of mortality was recorded as the number of mice dead per total number injected for a given dosage level after 24 hours. LD₅₀ values were computed according to the method of Litchfield and Wilcoxon (15).

TABLE I. Acute Toxicity of Histamine Diphosphate in Normal, Adrenalectomized and Pertussis-Treated Mice as a Measure of Histamine Sensitivity.

Treatment of mice	Histamine diphosphate, mg/kg, I.V.	Mortality		LD ₅₀ , mg/kg, I.V. (fiducial limits) 95%
		Ratio	%	
Normal (controls)	300	4/20	20	333 (309-367)
	350	9/20	45	
	400	16/20	80	
	550	16/16	100	
Adrenalectomized	150	0/6	0	315 (284-350)
	250	1/20	5	
	300	12/20	60	
	350	13/20	65	
	400	15/20	75	
Pertussis-treated	6	2/20	10	10 (8.8-12.3)
	8	8/20	40	
	10	8/20	40	
	14	14/20	70	

Results. Data presented in Table I reveal that pertussis-treated mice were 33 times more sensitive than normal mice to histamine injected intravenously, as indicated by LD₅₀ values of 10 and 333 mg/kg, respectively. However, the adrenalectomized mice were no more sensitive than normal mice to histamine injected intravenously, since the LD₅₀ values of 315 and 333 mg/kg, respectively, are almost identical.

Failure of adrenalectomized mice to exhibit sensitivity to histamine injected intravenously could not be due to incomplete adrenalectomy or growth of adrenal rests. A majority of the mice were considered to be completely adrenalectomized because: a. 63% of 71 adrenalectomized mice provided with Purina Chow and tap water died in 14 days; 94% of 16 adrenalectomized mice died in 14 days when provided with tap water and oatmeal, the latter having a lower sodium content than Purina Chow. b. Histamine was purposely injected 24 hr after adrenalectomy, thus eliminating the possibility of significant growth of adrenal rests. c. Furthermore, an experiment was made which confirmed the reports that adrenalectomized mice become sensitive to histamine injected intraperitoneally(10-14). Intraperitoneal injection of graded doses of histamine in 40 adrenalectomized mice revealed that they were 3 to 7 times as sensitive as normal mice.

Discussion. The evidence presented clearly indicates that pertussis-treatment greatly in-

creased sensitivity to histamine in the strain of mice used. It is clear that this strain of mice did not become sensitive to histamine injected intravenously after complete adrenalectomy. The failure of these adrenalectomized mice to exhibit sensitivity to histamine is not a unique finding since a CF-1 strain of mice was shown not to be sensitive after either injections of pertussis vaccine(10) or adrenalectomy(13). The important point indicated by the data herein presented is that the mice became sensitive to histamine after injection of pertussis vaccine, but not after adrenalectomy. This experiment therefore provides definite evidence that sensitivity to histamine which developed after treatment with pertussis vaccine cannot be ascribed to diminished function of the adrenal cortex and/or medulla, or to diminished action of hormones from the adrenal gland.

Although several authors have suggested that histamine sensitivity induced by pertussis vaccine is due to an injurious action of the vaccine on the adrenal glands, there has been no direct evidence presented to support the belief. If pertussis vaccine diminished the function of the adrenal medulla and cortex in mice one would expect the animals to become susceptible to various forms of stress. However, Kind(4) states that pertussis-treated mice did not become sensitive to injections of methacholine. Furthermore, the susceptibility to severe hypoxia(6) was accompanied by liver glycogen values which were not in-

dicative of abnormal adrenal cortical function. No evidence was presented to implicate abnormal adrenal function as the cause of hypoglycemia which developed in pertussis-vaccine treated mice(2). Other evidence exists which suggests the possibility that pertussis vaccine causes dysfunction of the adrenal glands. The sensitivity to histamine (injected I.P.) in pertussis-treated and adrenalectomized mice is partially or completely restored by proper treatment with cortisone (5) or a combination of cortisone and epinephrine(14). Nevertheless, the effects of these substances may be pharmacological in nature rather than specific physiological effects of replacement therapy after adrenal inactivation.

The authors are not aware of published data which prove that pertussis-vaccine alters the adrenal cortex as indicated by cholesterol content, ascorbic acid depletion, or other tests of adrenal function.

Summary. In a Swiss-Webster strain of mice, pertussis vaccine treatment increased the sensitivity to intravenously injected histamine 33 times, whereas surgical adrenalectomy had no significant effect on sensitivity to histamine. This finding, in addition to lack of direct evidence to the contrary, warrants the conclusion that *Hemophilus pertussis* vac-

cine enhances sensitivity of mice to histamine through some mechanism other than impairment of adrenal function.

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Diet and TEPA Therapy in Sarcoma-Bearing Rats.* (22151)

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Data have been presented to demonstrate that N, N', N'' triethylenephosphoramidate (TEPA) reduced food efficiency and growth of normal as well as tumor tissue in tumor-bearing rats(1). Given an optimum dosage and a susceptible tumor, however, reduction in growth of the tumor was greater than reduction in weight of normal tissues(2). These data also demonstrated that a high protein

diet, or a low casein diet supplemented with methionine, reduced the depleting effect of TEPA on normal tissue without altering the deleterious effect on the tumor(1,2). Other experiments have demonstrated that supplementing a low casein diet with a mixture of methionine and guanidoacetic acid or methionine and glycine resulted in increased protection of normal tissues to the depleting effects of the tumor(3). Further work has been done to determine the effect of these mixed supplementations upon chemotherapy of TEPA in tumor-bearing rats.

* Some of these results were read before the Am. Chem. Soc. Meeting in New York, Sept. 1954. These studies were supported by an Institutional Grant from the American Cancer Society.

TABLE I. Effect of Various Supplementations in Diet Containing 12% Casein upon TEPA Therapy (1 mg/Day/kg Body Wt). Food efficiencies were calculated as grams gain in body wt (tumor wt subtracted) per 100 g of food intake.

Diet	Food efficiency	Tumor wt, g	Gain in body wt/tumor
Avg data using 10 animals/group			
Casein 12% (no TEPA)	8.6	36.7	1.41 ± .26*
Casein 12% + TEPA	4.1	10.9	1.97 ± .39
+ glycine (Gl) + TEPA	4.4	9.7	2.29 ± .24
+ alanine (Al) + TEPA	4.2	10.7	2.02 ± .44
+ NH ₄ citrate (NH ₄) + TEPA	4.2	8.4	1.50 ± .86
+ methionine (M) + TEPA	5.4	8.4	3.24 ± .67
+ M + NH ₄ + TEPA	4.0	8.2	2.76 ± .80
+ M + Al + TEPA	5.7	13.3	3.00 ± .50
+ M + Gl + TEPA	5.4	7.2	3.61 ± .30
+ M + guanidoacetic acid + TEPA	8.1	7.2	5.64 ± 1.22
Casein 12% + TEPA	4.2	6.9	1.95 ± .67
+ M + TEPA	5.9	5.4	3.10 ± .96
+ M + NH ₄ + TEPA	6.2	7.1	3.37 ± .95
+ M + Gl + TEPA	6.0	7.1	3.15 ± .98
+ M + casein 1.5% + TEPA	6.1	7.5	3.04 ± .78
+ M + guanidoacetic acid + TEPA	9.6	4.6	6.57 ± 1.12
Avg data using 58 animals/group			
Casein 12% + TEPA	4.0	7.7	2.47 ± .59
+ M + TEPA	7.9	7.3	4.37 ± .74
+ M + guanidoacetic acid + TEPA	10.31	5.5	7.62 ± 1.00

* Stand. error.

Methods. Transplanting the sarcoma in the rat and measuring growth of the tumor have been described previously(1). The tumor developed most rapidly in rats fed a semi-synthetic diet containing 12% casein, a diet that has been reported in detail(1). The effect of various types of supplementation of the diet on growth of the body and the sarcoma in the presence of TEPA is reported in this paper. These supplements were 0.67% DL methionine and 0.67% DL methionine plus 0.67% guanidoacetic acid. These percentages and mixtures had been demonstrated to be optimum for growth of normal tissue in tumor-bearing rats(3,4) and for growth in weanling rats(5) fed 12% casein diet. Glycine (1.28%), alanine (1.53%), ammonium citrate (1.95%) and casein (1.5%) which contain nitrogen in amounts equivalent to that found in guanidoacetic acid (0.67%) were studied also as single supplements and combined with methionine. Food efficiencies were calculated as grams gained in body weight (tumor weight subtracted) per 100 g of food intake. TEPA was injected intraperitoneally as 1 mg/day/kg of body weight after tumor had reached approximately one g

in weight and was well established. Ten rats were used to determine the effect of each supplement on TEPA therapy.

Results. Data recorded in Table I illustrate food efficiencies, tumor weights and Δ body weight/tumor ratios approximately 3 weeks after transplantation of the sarcoma. The rate of increase in body with respect to the tumor is taken as one measure of the effect of the tumor on the body. At the end of 3 weeks the tumor had reached lethal proportions (36.7 g) in rats not given TEPA. The Δ body/tumor ratio was low (1.41) indicating a depleted body. The TEPA therapy (1 mg/day/kg body weight) was started in other groups of rats after the tumor had reached about one g in size and was well established. This therapy reduced growth of tumor to 10.9 g in rats fed 12% casein diet over this 3-week period but also reduced food efficiency and growth of the body of the animal. Supplementing the 12% casein diet with glycine, alanine or ammonium citrate did not alter results significantly. Supplementing with methionine, however, did increase food efficiency and rate of growth of the body with respect to the tumor in the

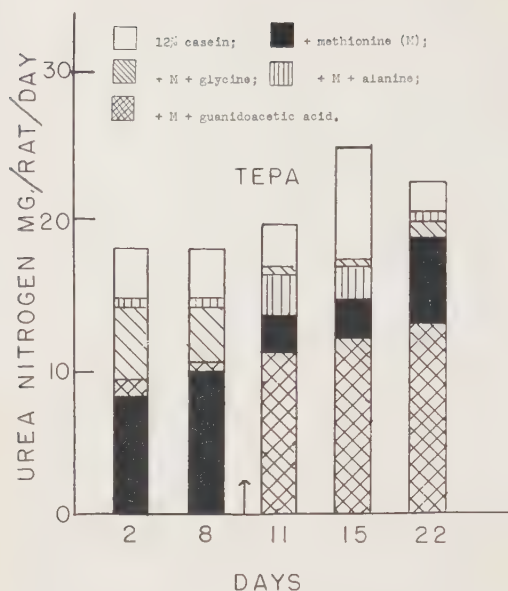


FIG. 1. Effect of different diets on excretion of urea nitrogen in sarcoma-bearing rats. All values start from base. TEPA therapy was instituted at time indicated by arrow.

presence of TEPA. Supplementing with methionine plus guanidoacetic acid increased food efficiency and rate of growth of the body still further. Adding an equivalent amount of nitrogen to guanidoacetic acid in the form of glycine, alanine, ammonium citrate or casein with the methionine did not have the marked effect noted with guanidoacetic acid, results which suggest a specific role for this acid.

The possibility that supplementation with a mixture of methionine and guanidoacetic acid could reduce catabolic activity of tissue proteins was suggested. The white bars in Fig. 1 record excretion of urea nitrogen in tumor-bearing rats fed 12% casein, data that illustrate the high excretion of urea nitrogen associated with a nutritionally malignant tu-

mor. The black bars illustrate reduction in excretion of urea nitrogen in rats fed the casein diet supplemented with methionine. The bars with slanted lines record excretion in the presence of methionine plus glycine while bars with vertical lines record excretion of urea nitrogen in the presence of methionine plus alanine. Adding glycine or alanine increased excretion of urea, probably reflecting the catabolism of these amino acids. The bars with crossed lines illustrate excretion of urea nitrogen in tumor-bearing animals fed the casein diet supplemented with both methionine and guanidoacetic acid. Thus methionine plus guanidoacetic acid reduced excretion of urea nitrogen markedly during TEPA therapy, a reduction that may be correlated with better gain in body weight. These results emphasize the importance of combining nutritional therapy with chemotherapy.

Summary. Data have been presented to demonstrate that TEPA reduced food efficiency and growth of body weight as well as tumor weight in sarcoma-bearing rats fed a 12% casein diet. Supplementing with methionine and guanidoacetic acid increased food efficiency and growth of the body without increasing the growth of the tumor. This increase in growth of the body is correlated with reduction in excretion of urea.

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